MOLECULAR CLONING AND FUNCTIONAL ANALYSIS OF *9-CIS* EPOXYCAROTENOID DIOXYGENASE GENE FAMILY OF ARABIDOPSIS

BY

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Abscisic acid (ABA) regulates seed dormancy and germination, and gene expression in response to various environmental stresses. In plants, ABA is synthesized from epoxycarotenoid precursors that are localized exclusively in the membranes of plastids. Oxidative cleavage of epoxycarotenoids catalyzed by *9-cis* epoxycarotenoid dioxygenase (NCED) is the key regulatory step in the ABA biosynthetic pathway. Previous work in maize and other species has shown that NCED is encoded by a complex gene family. The Arabidopsis genome sequence revealed nine putative genes showing sequence homology to NCED. These are designated 9-cis epoxycarotenoid dioxygenase (*AtNCED*). The *AtNCED2, 3, 5,* and *9* genes show a strong sequence identity to the *Vp14* from maize as well as *NCEDs* from other species, and are thus potentially the *NCED* homologs involved in ABA biosynthesis. The *AtNCED1, 4*, and *6* genes have moderate homology to *NCED* and have unknown functions. The *AtNCED7* and *AtNCED8* are more

closely related to β-carotene dioxygenases of animals that catalyze retinal biosynthesis. Northern analysis revealed that the *AtNCEDs* are differentially expressed in leaves and roots, and in some cases induced by water stress. *In vitro* chloroplast import experiments showed that AtNCED2, 4, and 5 proteins are differentially localized within chloroplasts. The AtNCED2 is partially localized in the stroma, and partially associated with the thylakoid membranes. In contrast, AtNCED4 is localized only in the stroma, whereas AtNCED5 is associated exclusively with thylakoid membranes. The imported mature AtNCED5 was larger than its translation precursor suggesting modification post-import. The differential expression, localization, and post-import modification of the *AtNCEDs* suggest that ABA biosynthesis is regulated both at the transcriptional and post-translational levels.

AtNCED2 is 90% similar to VP14 of maize. To study the regulation of ABA biosynthesis and the *AtNCED2* gene, *AtNCED2* promoter-GUS, sense overexpression, and antisense expression transgenic plants were constructed. The *AtNCED2* promoter-GUS transgenic lines showed GUS staining at the base of mature lateral roots, a narrow ring of cells in the root cap, and pericycle and cortex cells surrounding the lateral root initials. Auxin treatment induced lateral root formation and also strongly induced GUS staining, suggesting that ABA may be involved in lateral root initiation. Transgenic plants overexpressing the *AtNCED2* showed phenotypes of inhibited growth, fewer lateral roots, male sterility, and anthocyanin accumulation. No obvious phenotype was observed in antisense lines.

We also screened knockout mutants of the *AtNCED* gene family. Two knockout mutants of *AtNCED2* and one mutant of *AtNCED5* have been identified and narrowed

down to individual plants. Positive candidates have been identified for all the other family members, and further screening is in progress.

CHAPTER 1
LITERATURE REVIEW

Functions of Abscisic Acid in Plants

Abscisic acid (ABA) regulates multiple physiological processes including seed dormancy and germination, stomatal aperture, and gene expression in response to environmental stresses. Much of the information concerning ABA function has come from characterization of effects of exogenous application of ABA to plants and from analysis of ABA-deficient mutants.

Regulation of Seed Maturation, Germination, and Desiccation Tolerance

Abscisic acid (ABA) regulates essential processes during seed development including induction of seed dormancy, accumulation of reserve proteins, and acquisition of desiccation tolerance. Normally, ABA levels start to increase in the middle phase of seed development and peak at the time of maximum dry seed weight, and then decrease to lower levels toward seed maturity (Koornneef and Karssen 1994, Chandler and Robertson 1994). Immature embryos excised from seeds are able to germinate readily when placed in culture, and this process can be suppressed by applying ABA in the culture (reviewed in Quatrano 1987). Moreover, the endogenous ABA concentration of embryos of mid-maturation correlates well with their ability to germinate, embryos containing less ABA are easy to germinate (Rock and Quatrano 1995). Thus, ABA plays a key role in arresting embryo growth.

The function of ABA in suppressing precocious germination and inducing dormancy of developing seeds also was confirmed by studying mutants defective in either ABA content or ABA sensitivity. Such mutants have been isolated and analyzed mostly in maize and Arabidopsis. Seeds of ABA deficient mutants from maize display a *viviparous* phenotype; that is, seeds precociously germinate while still attached to the mother plant (Robertson 1955). In Arabidopsis, seeds of ABA biosynthetic mutants are typically nondormant. Normally, cold or light treatments are required to break dormancy of Arabidopsis seeds. The ABA deficient mutants relieve the requirement for dormancy breaking treatments. However, in contrast to the *viviparous* seed phenotype of maize mutants, even severe ABA-deficient mutants of Arabidopsis produce seeds that are non-viviparous and desiccation tolerant, suggesting that Arabidopsis seed development is less dependent on ABA signaling (McCarty 1995).

The late stage of seed development features accumulation of storage proteins and late-embryogenesis abundant (LEA) proteins. The LEA proteins are thought to participate in desiccation tolerance (Skriver and Mundy 1990, Dure 1993). The expression of these proteins is promoted by ABA. Transcripts of storage and LEA proteins can be upregulated precociously when immature embryos are placed in medium containing ABA (Parcy et al. 1994). Moreover, ABA-deficient mutants of Arabidopsis and maize fail to accumulate certain storage or LEA proteins (Paiva and Kriz 1994). This evidence supports the role for endogenous ABA in developmental expression of these genes in seeds.

Several genes encoding LEA proteins have been cloned. Functional dissection of their promoter regions identified several cis-elements involved in ABA responses. The G-

box ACGT core motif, designated as ABRE's (ABA Response Elements) were found in Rab16 LEA gene from rice, the Em LEA gene of wheat, and several other LEA genes from different species. A second type of ABRE is the Sph element found in C1 gene of maize. The C1 is a regulator of anthocynin biosynthesis in seeds, and the Sph element is essential for ABA induction of C1 gene expression during seed development (Kao et al. 1996; Hattori et al. 1992).

Regulation of Stomatal Aperture

Abscisic acid mediates plant responses to water stress by triggering stomatal closure to limit water loss through transpiration. The stomatal pore is composed of a pair of guard cells, which by their shrinking and swelling control the closure and opening of a stomatal pore. Applying ABA to leaves promotes stomatal closure and inhibits opening (Assmann 1993). The ABA deficient mutants display a wilty phenotype due to failure of stomatal closure (Koorneef et al. 1982, Leon-Kloosterziel et al. 1996). This evidence supports a function of ABA in regulating stomatal closure.

The mechanism of how guard cells perceive ABA and how the ABA signal is transduced is not fully understood. Despite extensive research efforts, the nature and location of the guard cell ABA receptor is still elusive. Conflicting evidence indicates that ABA receptors are located on the plasma membrane of guard cells as well as intracellularly. A plasma membrane localized ABA receptor is supported by evidence that ABA microinjected directly into the cytosol of Commelina guard cells failed to trigger stomatal closure (Anderson et al. 1994). However, another independent study showed that stomatal closure could be triggered by injection of ABA into cytosol of

Commelina guard cells (Allan et al. 1994), supporting an intracellular localization of the ABA receptor.

Although little progress has been made in isolating the guard cell ABA receptor, great advances have been made in identifying downstream components. Using patch clamp techniques, three types of ion channels in the plasma membrane of guard cells have been implicated in controlling stomatal aperture. These are inward- and outward-rectifying K^+ channels, and anion channels. Their activities are modulated by ABA (Armstrong et al. 1995). In response to increased ABA, the first detected electrical change is an influx of positive charge into guard cells, which is contributed by Ca^{2+} and H^+ (Thiel et al. 1992). These cations are proposed to act as secondary messengers that activate Ca^{2+} sensitive and voltage sensitive anion channels, which in turn mediate the long-term depolarization and a large efflux of anions across the plasma membrane (Schroeder and Hagiwara 1990). The efflux of anions drives K^+ efflux through an outwarding-rectifying K^+ channel (Pei et al. 1997), causing loss of turgor of guard cells and stomatal closure. The ABA also can inhibit the K^+ influx into guard cells by inhibiting inward-rectifying K^+ channels. When ABA levels decrease, K^+ uptake is mediated by inward-rectifying K^+ channels causing stomatal opening (Blatt 1992).

In addition to ion channels, Ca^{2+}, H^+, and phosphorylation and dephosphorylation are identified as components involved in ABA signaling. Cytosol Ca^{2+} levels ($[Ca^{2+}]_{cyt}$) increase in response to ABA. As mentioned above, Ca^{2+} influx is the first detected signal and acts as the secondary messenger contributing to membrane depolarization. Release of caged Ca^{2+} into guard cells is sufficient to induce stomatal closure and this event mimics the effect of ABA on ion channels (Gilroy et al. 1990).

Another event that correlates with ABA-induced stomatal closure is cytosolic pH. Microinjecting BCECF, a pH indicator, into guard cells of *Paphiopedilum tonsum* has been used to monitor pH change in response to ABA. An ABA-induced increase of cytosolic pH ranging from 0.04 to 0.3 pH units was observed (Irving et al. 1992). It was also found that activities of both outwarding and inwarding K^+ channels were modulated by pH (Thiel 1997).

Protein phosphorylation and dephosphorylation also are involved in ABA signaling. The ABA-insensitive mutants *abi1* and *abi2* are insensitive to ABA induced stomatal closure. ABI1 and ABI2 encode protein phosphatases 2C homologs indicating a role for protein phosphorylation/dephosphorylation in ABA signaling (Leung et al. 1994, 1997). Both mutants are impaired in ABA elevation of $[Ca^{2+}]_{cyt}$ and ABA induced activation of a slow anion channel (Allen et al. 1999). Transgenic plants with the *abi1-1* dominant mutant allele exhibit insensitivity of guard cell K^+ inwarding- and outwarding-channels to ABA. Normal sensitivity of K^+ channel activity and stomatal closure were recovered when protein phosphorylation antagonists were applied (Armstrong et al. 1995). These results indicated that an ABA-evoked signal is transduced into guard cells by a phosphatase/kinase pathway.

The source of ABA that induces stomatal closure in leaves is unknown. The closing of stomata usually occurs within 5 minutes of imposed water stress before any detectable change of ABA content in the leaves (Cornish and Zeevaart 1985). One explanation is that ABA is mobilized and accumulated into guard cells from neighbor cells, or highly localized de novo ABA synthesis is induced in guard cells immediately upon water stress, or both events happen in the guard cells at the same time. Experiments

using a single guard cell system have demonstrated that upon water deficit, a fast increase of ABA concentration within a single guard cell correlates with the stomatal closure; however, the source of ABA was not addressed in this study (Harris and Outlaw 1991).

Adaptation to Environmental Stresses

Abscisic acid mediates plant responses to various adverse environmental stresses, including drought, cold, salt, mechanical wounding and pathogenic infections (Ingram and Bartels 1996). Many physiological studies have shown that endogenous ABA levels increase as a result of environmental stresses. As discussed before, increased ABA levels stimulate stomatal closure to reduce water loss through transpiration. In addition, plants develop freezing tolerance when treated with ABA under nonacclimating conditions (Lang et al. 1994). Exposure of plants to low, nonfreezing temperatures induce a process known as cold acclimation, in which plants increase their cold tolerance. The ABA-deficient mutants of Arabidopsis are freezing intolerant compared to wildtype and this defect can be complemented by exogenous ABA (Ingram and Bartels 1996).

A wide range of genes induced by drought, cold, and salt are affected in ABA deficient mutants. The expression of these proteins can be induced by exogenous applied ABA in unstressed conditions (Skriver and Mundy 1990, Mantyla et al. 1995). Many LEA proteins that are abundantly expressed in the final desiccation stage of seed development also are expressed in vegetative tissues in response to dehydration or ABA (Ingram and Bartels 1996). Evidence suggests that a structural motif of LEA protein is responsible for its function in dehydration tolerance. The LEA proteins have a biased amino acid composition. They are rich in glycine and glutamine that result in highly hydrophilic protein properties. The LEA proteins have a randomly coiled structure that is

consistent with their role in binding water, and therefore helping cells maintain stability with minimum water. Also, because of their high concentrations in the cell and biased amino acid composition, the LEA proteins do not appear to function as enzymes (Baker et al. 1988).

The proteinase inhibitor II (*Pin2*) gene family is well known for responding to mechanical wounding. Endogenous ABA levels increase upon wounding. Exogenous applied ABA induces *Pin2* gene expression in a manner analogous to wounding. Wounding induced *Pin2* gene expression is abolished in ABA-deficient mutants (Pena-Cortes and Willmitzer 1995).

The Abscisic Acid-Related Mutants in Higher Plants

Mutants that affect ABA responses in plants can be classified into ABA-deficient mutants and ABA signal transduction mutants.

Abscisic Acid-Deficient Mutants

ABA deficient mutants are impaired in the ABA biosynthetic pathway, and in appropriate circumstances can be rescued by exogenous applied ABA. These mutants provide a valuable tool to study the physiological function of ABA in seed development and vegetative growth. These mutants also contributed extensively to the elucidation of the ABA biosynthetic pathway.

The ABA deficient mutants were identified in variety species (Table 1) including *viviparous* mutants of maize, *aba* mutants of Arabidopsis, *flacca, sitiens, and notabilis* of tomato, *aba1* of *Nicotiana plumbagiinifolia*, *wilty* of pea, and *droopy* of potato (reviewed

in Giraudat 1995). Several of the maize mutants *vp2, vp5, vp7, vp9* are blocked in the early steps of carotenoid biosynthesis suggesting that ABA is synthesized from

Table 1. Abscisic acid deficient mutants

Impaired step	Mutant	Phenotype	Species	Reference
Zeaxanthin epoxidation	*aba1*	wilty, nd,	Arabidopsis	Koornneef 1982, Rock 1991
	aba2	wilty, nd	tobacco	Main 1996
Xanthophyll cleavage	*vp14*	vp	maize	Tan 1997, Schwartz 1997
	notabilis	wilty	tomato	Taylor 1988, Burbidge 1999
Xanthoxin oxidation	*aba2*	wilty, rd	Arabidopsis	Leon 1996, Schwartz 1997
ABA-ald oxidation	*aba3*	wilty, rd	Arabidopsis	Leon 1996, Schwartz 1996
	ckr1	wilty	tobacco	Parry 1991
	flacca	wilty	tomato	Parry 1988
	sitten	wilty	tomato	Parry 1988
	droopy	wilty	potato	Duckham 1989

nd=nondormant, rd=reduced dormancy, vp=viviparous

carotenoid precursors. Consistent with the carotenoid origin of ABA, *aba1* of Arabidopsis is impaired in the conversion of zeaxanthin to violaxanthin (Rock and Zeevaart 1991). The *aba2* gene of tobacco was isolated by heterologous *Ac* tagging and it was shown to encode the zeaxanthin epoxidase in ABA biosynthesis. That the *aba2* of tobacco is an ortholog of Arabidopsis *aba1* was shown by using the *aba2* gene of tobacco

to complement the *aba1* phenotype. The maize *vp14* and tomato *notabilis* mutants are impaired in the oxidative cleavage of 9-cis xanthophylls. The *aba2* and *aba3* of Arabidopsis are impaired in the last two steps of ABA biosynthesis, *aba2* is blocked in the conversion of xanthoxin to ABA-aldehyde and *aba3* is blocked in the conversion of ABA-aldehyde to ABA. The tomato *flacca* and *sitiens*, potato *droopy* and tobacco *aba1* mutants are all blocked in the last step of ABA-aldehyde to ABA.

Abscisic Acid-Responsive Mutants

The well-characterized ABA-insensitive mutants are Arabidopsis *abi1, abi2, abi3, abi4, abi5,* and maize *vp1*. The *abi1, abi2, abi3* mutants were isolated by selecting for germination of EMS mutagenized seeds on concentrations of ABA that inhibit normal seed germination. The Arabidopsis *abai3* and maize *vp1* mutants display common seed specific phenotypes of which seeds are non-dormant and desiccation intolerant. However, *abi3* mutant seeds are not viviparous like *vp1,* indicating that seed development in Arabidopsis is less dependent on ABA signaling (McCarty 1995). The *vp1* of maize also blocks anthocyanin biosynthesis in seeds. Cloning of *Vp1* and *ABI3* showed that they encode homologous proteins that function as transcription factors (McCarty et al. 1991, Giraudat et al. 1992). *In vitro* transient assays showed that VP1 and ABA synergistically activate C1, Em expression (Kao et al. 1996, Hoecker et al. 1995), and VP1 represses α-amylase expression (Hoecker et al. 1995). The B3 domain of VP1 was found to have cooperative DNA binding activity to the Sph element of the C1 promoter (Suzuki et al. 1997).

In addition to the reduced seed dormancy phenotype, *abi1* and *abi2* also showed an increased tendency to wilt. This phenotype was correlated with abnormal stomatal

aperture (Leung et al. 1994). Cloning of the *ABI1* gene showed that it encodes a 2C type protein phosphatase (Meyer et al. 1994). Cloning of *ABI2* showed that it encodes a similar protein. Both ABI1 and ABI2 were suggested to be components involved in many aspects of ABA responsiveness (Leung et al. 1997).

The Biosynthetic Pathway of ABA in Higher Plants

Abscisic acid is a sesquiterpene ($C_{15}H_{20}O_4$). Although its structure was elucidated in 1965 (Addicott 1965), study of its biosynthetic pathway has been quite a challenge for researchers. First of all, ABA is usually present in plant tissue at a very low concentration, ranging from 10^{-8} M to 10^{-7} M. The ABA levels reach higher than 10^{-6} M only in water-stressed leaves and developing seeds. A second difficulty was that the radioactive labeled putative precursors incorporate poorly into ABA (Walton and Li 1995).

The ABA biosynthetic pathway in fungi was discovered due to the finding that *C. rosicola* secretes a considerable amount of ABA into its growth medium. In this so called direct pathway, 1'-deoxy-ABA or 1', 4'-t-diol-ABA is the immediate precursor of ABA. However, in higher plants, ABA is synthesized by an indirect pathway in which ABA is a cleavage product of xanthophylls.

The Indirect Pathway of ABA Biosynthesis in Higher Plants

In higher plants, ABA is synthesized from oxidative cleavage of xanthophylls to produce the immediate ABA precursor, xanthoxin. Xanthoxin is further oxidized to produce ABA. The evidence that ABA is synthesized from cleavage of xanthophylls accumulated from the following observations and experiments.

1. Several maize mutants (*vp2*, *vp5*) that lack the ability to synthesize carotenoids and display seedling lethal phenotype due to photobleaching are also ABA deficient. Inhibitors of carotenoid biosynthesis, such as fluridone or norflurazon, also can block the accumulation of ABA production (Henson 1984, Moore and Smith 1984). These observations suggest that carotenoids are involved directly in ABA biosynthesis.
2. When incubated with ^{18}O, water-stressed bean and *Xanthium* leaves rapidly incorporated ^{18}O into the carboxyl group of ABA, indicating that ABA in water-stressed leaves is synthesized from a large precursor pool that already contains oxygen on the ring of the ABA. This experiment suggested that xanthophylls are ABA precursors because they exist in high concentration in leaves, and the structure of the end group of xanthophylls having 3-hydroxy- and 5,6-epoxy groups corresponds to the 4'- and 2',1'-positions of ABA (Creelman and Zeevaart 1984).

   To test whether violaxanthin served as an ABA precursor, plant leaves were treated with ^{18}O so that oxygen in the epoxy groups was replaced by ^{18}O (Li and Walton 1987). The plant leaves then were water-stressed and violaxanthin and ABA were extracted and analyzed. The results of mass spectrometry showed that violaxanthin contained ^{18}O in its epoxide group and the ABA produced during water stress was labeled by ^{18}O in its 1'-hydroxyl group. Therefore, the ^{18}O on the ABA 1'-hydroxyl group would be derived from the epoxide group of violaxanthin. However, one limitation of this study was that violaxanthin accounted for only a quarter of the ABA produced.
3. Because the xanthophyll levels in higher plants are many times higher than ABA, it is difficult to establish the relationship between xanthophyll precursors and the ABA

produced. Li and Walton (1990b) used etiolated bean seedlings in which xanthophyll levels are greatly reduced, but ABA production levels during water stress reached about same level as in green seedlings. Xanthophyll levels, ABA, and its metabolites were analyzed in these materials after a short water stress. There was 1:1 stoichiometry between the loss of violaxanthin and neoxanthin and the production of ABA and its metabolites, phaseic acid (PA), and dihydrophaseic acid (DPA).

4. The Arabidopsis ABA-deficient mutant *aba1* is impaired in epoxidation of zeaxanthin to antheraxanthin and violaxanthin, causing zeaxanthin accumulation and very low levels of violaxanthin and neoxanthin (Rock and Zeewaart 1991).

With a combination of genetic, molecular, and biochemical approaches, several genes on ABA biosynthetic pathways in higher plants have been cloned. The *ABA2* gene of *Nicotiana plumbaginifolia* was cloned by using heterologous transposon tagging Ac. The *ABA2* is the homolog of the *ABA1* gene of Arabidopsis and it encodes zeaxanthin epoxidase (Marin et al. 1996). The *Vp14* of maize was cloned by transposon tagging and it encodes a specific *9-cis* xanthophyll cleavage enzyme (Tan et al. 1997, Schwartz et al. 1997). Hence, Zeaxanthin is epoxidated to produce all *trans* violaxanthin, which is then isomerized to produce 9-*cis* violaxanthin and 9-*cis* neoxanthin. Because *9-cis* neoxanthin is much more abundant than *9-cis* violaxanthin, it is considered to be the primary source of ABA. The 9-*cis* xanthophylls are oxidatively cleaved to produce xanthoxin, and they are further oxidized to produce ABA (Fig. 1-1). The importance of the double bond isomerization step converting all *trans* violaxanthin to *9-cis* violaxanthin is indicated by the fact that VP14 can only use *9-cis* xanthophyll as substrates (Schwartz et al. 1997) and

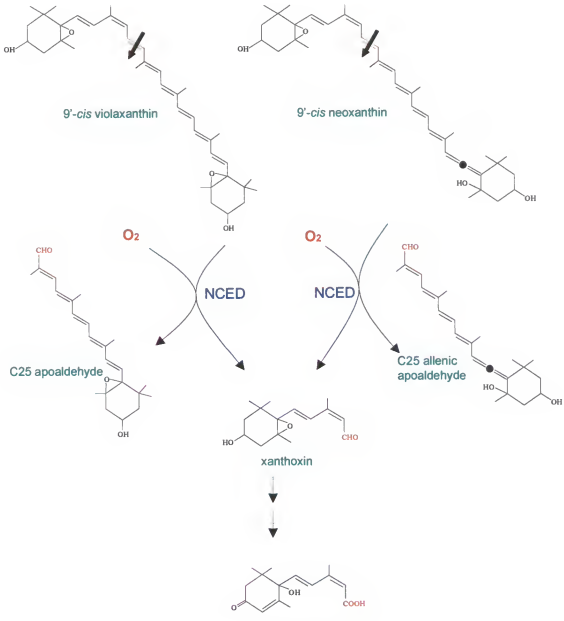

Fig. 1-1. The ABA biosynthetic pathway in higher plants. Oxidative cleavage of epoxycarotenoids is the first committed and the key regulatory step. The dotted arrows reflect multiple steps involved in the final formation of ABA. The solid arrows indicate the 11, 12 -double bond cleaved by 9-cis epoxy carotenoid dioxygenase (NCED).

all *trans*-xanthoxin can not be converted to biologically active ABA by cell free extracts (Zeevaart and Creelman 1988).

The Rate-Limiting Step of Abscisic Acid Biosynthesis

Biochemical evidence has indicated that oxidative cleavage of *9-cis* xanthophylls is the key regulatory step of ABA biosynthesis in higher plants (Sindhu and Walton 1987, Creelman et al. 1992). The epoxy-carotenoid precursors of ABA are distributed widely in plant tissues and are in high concentration relative to ABA, so the zeaxanthin epoxidase is unlikely to be the regulated step. The enzyme activities of the last two steps that convert xanthoxin to ABA are constitutively active in most tissues (Li and Walton 1990a). This biochemical evidence led to the hypothesis that oxidative cleavage of 9-cis xanthophylls is the key regulatory step in ABA biosynthetic pathway.

Cloning of *Vp14* from maize made it possible to study the *9-cis* epoxycarotenoid dioxygenase in other species. Families of *Vp14*-related genes have been reported in maize, tomato, bean and Arabidopsis. Emerging evidence has shown that families of *Vp14*-like genes provide overlapping sources of ABA with differential tissue specific expression, and *Vp14* related gene families may provide actual biological control of ABA biosynthesis in higher plants.

VP14 of maize. *Viviparous 14* (*vp14*) of maize was identified as a *viviparous* mutant in a Robertson's *Mutator* strain. The embryos of *vp14* mutant start to germinate while still attached to the mother ear. The seedlings of *vp14* show no discernible phenotype. *Viviparous14* has reduced levels of ABA in embryos and in water-stressed leaves. Analysis of intermediates of ABA pathway in v*p14* embryos showed that *vp14* has normal levels of all-trans violaxanthin, 9-cis violaxanthin, and 9-cis neoxanthin; and

that *vp14* cell-free extract can convert xanthoxin to ABA, indicating that *vp14* is impaired in the cleavage step (Tan et al. 1997).

The *Vp14* was cloned by transposon tagging. The amino acid sequence of *Vp14* shares homology to lignostilbene dioxygenase (LSD) of *Pseudomonas Paucimobilis* that catalyzes oxidation cleavage of the central double bond of lignostilbene. This reaction is similar to the oxidative cleavage reaction proposed in the ABA biosynthetic pathway (Tan et al. 1997). The recombinant VP14 protein expressed in E. coli specifically catalyzes the oxidative cleavage of *9-cis* xanthophylls to form C25 apo-aldehydes and xanthoxin. The reaction requires oxygen, ferrous iron, and a detergent (Schwartz et al. 1997).

The expression of *Vp14* mRNA is detected in the developing embryos, roots and water-stressed leaves, but not in the leaves under normal growing conditions. The normal levels of endogenous ABA in leaves and roots and the normal plant phenotype of *vp14* mutant indicated that *Vp14* only is responsible for a subset of ABA synthesized in vegetative tissues. However, detached leaves of *vp14* mutant seedlings have a markedly increased rate of water loss relative to the wild type, and this difference is detected within 5 minutes after leaf excision. These results suggest that the *vp14* mutant is unable to rapidly induce stomatal closure after water stress and that VP14 protein may be involved in stomatal regulation in response to leaf excision.

Water stress could induce stomatal closure within five minutes, and it was suggested that bulk ABA synthesis may occurs too slowly to induce these rapid stomatal responses. Therefore, the ABA source for stomatal closure may come from rapidly localized ABA synthesis around stomata complex or rapid redistribution of a pre-existing

ABA pool. The phenotype of the *vp14* mutant affecting stomatal closure may be explained by highly localized ABA pool in leaves that specifically control stomatal closure. The ABA in this pool may result from highly localized *Vp14* expression in leaves that cannot be detected by northern analysis. Alternatively, ABA may be remobilized from roots, which is consistent with root expression of *Vp14* (Tan et al. 1997).

Because epoxycarotenoids, the substrate of ABA, are localized exclusively in the membranes of chloroplasts and other plastids (Zeevaart and Creelman 1988, Walton and Li 1995), it has been suggested that initial steps of ABA biosynthesis are localized inside chloroplasts. Using *in vitro* import assay, it was demonstrated that VP14 is imported into chloroplasts, where it is partly bound with the thylakoid membranes and partly soluble in the stroma. It was shown that an amphipathic helix region downstream of the transit peptide of VP14 is required but not sufficient for targeting VP14 to the thylakoid membranes (Tan et al. unpublished data).

9-*cis* epoxycarotenoid dioxygenase of tomato (LeNCED). The *Notabilis* (*not*) mutant of tomato has a mild wilty phenotype, and it was suspected to be impaired in the oxidative cleavage step of the ABA biosynthetic pathway. The corresponding gene was cloned by screening a wilt-related tomato cDNA library using a PCR fragment amplified from degenerate primers based on conserved sequences of VP14 and related dioxygenases from different species. The cloning of *LeNCED1* provides the evidence of the cleavage enzyme in a dicot species. The *not* mutant has a single A/T base pair deletion that creates a premature stop codon and a severely truncated protein. The *not* allele therefore is probably a null mutant. However, the mild plant phenotype of *not*

mutant once again shows there is a substantial genetic redundancy for the cleavage step. Like VP14, LeNCED also has a transit peptide and an amphipathic helix characteristic region in its N-terminal (Burbidge et al. 1999).

9-cis epoxycarotenoid dioxygenase of bean (pvNCED). The *pvNCED1* gene was cloned from wilted bean by screening a cDNA library using a PCR fragment amplified from degenerate primers based on conserved sequence of Vp14 and *LeNCED1* (Qin et al. 1999). The recombinant protein pvNCED1 catalyzes the cleavage of 9-cis-neoxanthin and 9-cis violaxanthin. In detached bean leaves, the *pvNCED1* transcripts and pvNCED1 protein levels were largely induced and the increase precedes the accumulation of ABA. Similar correlation among *pvNCED1* mRNA, protein and ABA level also were observed in roots. However, the increased level of ABA is much less pronounced in roots compared to leaves presumably due to the smaller carotenoid precursor pool in roots. PvNCED1 is imported into chloroplasts. However, different from VP14, PvNCED1 is mostly associated with thylakoid membranes.

CHAPTER 2
MOLECULAR CLONING AND EXPRESSION ANALYSIS OF
9-CIS EPOXYCAROTENOID DIOXYGENASE-LIKE
GENES IN ARABIDOPSIS

Abstract

Abscisic acid (ABA) is synthesized from carotenoids in plants. Oxidative cleavage of *9-cis* epoxycarotenoids by *9-cis* epoxycarotenoid dioxygenase (NCED) is the first committed and the key regulatory step in the ABA biosynthesis. The *NCED* has been reported to be encoded by a complex gene family in several different plant species. Arabidopsis genome revealed nine putative genes showing different degrees of sequence homology to *NCED*. *AtNCED2, 3, 5, 9* have about 65% sequence identity to VP14 at the amino acid level, thus are very likely to function as NCED's in Arabidopsis. Northern analysis showed that these four *AtNCEDs* plus *AtNCED1* and *AtNCED4* are differentially expressed in leaves and roots, and induced by water stress. The expression pattern of *AtNCEDs* suggests that there are overlapping sources of ABA during plant growth and development, and in response to environmental stresses. Moreover, *AtNCEDs* may have overlapping but distinctive roles in regulating ABA biosynthesis.

Introduction

Abscisic acid (ABA) is synthesized from precursor carotenoids in plants. The *9-cis* neoxanthin and *9-cis* violaxanthin are oxidatively cleaved to produce the immediate ABA precursor, xanthoxin. Xanthoxin is further oxidized to produce ABA. The oxidative

cleavage of *9-cis* epoxycarotenoids by *9-cis* epoxycarotenoid dioxygenase (NCED) is the first committed and key regulatory step. The NCED is encoded by a complex gene family. Because biosynthesis of plant hormones is highly regulated by developmental and environmental factors, it has been proposed that a differentially regulated gene family may be a common mechanism in regulating plant hormonal biosynthesis. The key regulatory enzyme of ethylene biosynthesis, ACC synthase, is also encoded by a differentially regulated gene family (Liang et al. 1992). Families of *Vp14*-related genes have been reported in maize, tomato, bean, cowpea, and avocado. The above *NCED* genes are regulated by water stress at the level of transcription, supporting that ABA biosynthesis is regulated by the 9-cis epoxycarotenoid cleavage reaction (Tan et al. 1997, Burbidge et al. 1999, Qin et al. 1999, Iuchi et al. 2000, Chernys et al. 2000). We studied ABA biosynthesis by analyzing transcriptional expression of *AtNCED* genes in non-stressed leaves, roots, and stressed leaves and roots.

Materials and Methods

Stress Treatment

Leaves and roots were harvested from germination plates and left on the bench to allow the tissues to lose 15% of their fresh weight. The materials then were sealed in plastic bags and kept in the dark for 6 hours. Unstressed leaves and roots were sealed directly in bags and kept in the dark for 6 hours.

Northern Analysis

Total RNA was used for Northern blots of *AtNCED1, 3, 4, 5, 9* and *actin*; and mRNA was used for *AtNCED2* and *Vp14* of maize to enable to detect expression. The

full length cDNA of *AtNCED1* (has multiple introns) and *Vp14*, and full length genomic DNA of *AtNCED2, 3, 4 5, 9* (all are intronless) were used as probe. The cDNA of *AtNCED1* was obtained from the Arabidopsis stock center. The full length coding regions of *AtNCED2, 3, 4, 5, 9* (all these genes are intronless) were generated by PCR using genomic DNA as template.

Total RNA was extracted from leaves, stressed leaves, roots, and stressed roots using Trizol reagent (BRL) according to the manufacturer's instructions. Poly(A)$^+$-enriched RNA was purified using PolyATtract (Promega) according to the manufacturer's instructions and was quantified spectrophotometrically. Twenty µg of total RNA or 1 µg of Poly(A)$^+$-RNA was separated in 1.2% agarose gel containing formaldehyde (Sambrook et al. 1989). The gels were blotted onto nylon membranes, and the membranes were UV-linked and hybridized according to the method of Church and Gilbert (1984). The membranes were washed in a buffer containing 40 mM sodium phosphate (pH 7.2), and 1% SDS at 65°C for two hours. The probes were radiolabeled with random Primer DNA Labeling System (BRL) in the presence of ^{32}P-α-dCTP (Dupont).

Results

Cloning of *AtNCED1*

The A*tNCED1* gene was identified from an Arabidopsis EST database homology search. The EST sequence showed homology to *Vp14* and was used as a probe to screen an Arabidopsis genomic EMBL-3 library (Clontech). Clones containing an *AtNCED1* genomic sequence were isolated. The AtNCED*1* gene contains multiple introns and a mini exon of only 15 bp. The nearly full length *AtNCED1* cDNA was obtained from the

Arabidopsis stock center. It is predicted to encode a protein of 538 amino acids with 38% identity and 60% similarity to VP14 (Fig. 2-1). The AtNCED1 protein lacks an apparent transit peptide, but has an N-terminal amphipathic helix (AMP) sequence (refer to Fig.3-2 in chapter 3).

Cloning of *AtNCED2*

Degenerate primers (forward primer TTC/TGAC/TGGIGAC/TGGIATGGT, reverse primer ACIG/CCA/GAAA/GTCA/GTGCATCAT , where I=inosine) were designed based on the regions of conserved sequence between VP14 and its homolog in bacteria, lignostilbene dioxygenase (LSD). A 600 bp PCR fragment was amplified using Arabidopsis genomic DNA as a template. This PCR product showed high sequence similarity to *Vp14*, and thus was used as a probe to screen an Arabidopsis thaliana genomic EMBL-3 library (Clontech). About 10 positive clones were isolated from the library. Sequence of a partial genomic clone revealed an intronless open reading frame that is highly homologous to *Vp14*. All the other positive clones turned out to be *AtNCED2* based on restriction map analysis and partial sequence analysis. The deduced amino acid sequence of *AtNCED2* contains 583 amino acids and has a 66% identity and a 90% similarity to VP14 (Fig.2-1). Like *Vp14*, the *AtNCED2* gene appears to be intronless. The AtNCED2 protein also bears an N-terminal extension that resembles a chloroplast transit peptide. There is also a region of 19 amino acids resembling an AMP helix downstream of its N-terminal extension (refer to Fig. 3-2 in chapter 3).

```
VP14    : ------------------------------------------------------------------- :   -
AtNCED2 : ------------------------------------------------------------------- :   -
AtNCED3 : MHLSKCERSQVSTLNHNNITHTPFFFLFFSQSTLFNSLYNPLTPFPLSVTDSTRGHTDGIHLSSYPP :  67
AtNCED5 : ------------------------------------------------------------------- :   -
AtNCED9 : -------------------------------------------------------------MTIITI :   6
AtNCED6 : ------------------------------------------------------------------- :   -
AtNCED4 : ------------------------------------------------------------------- :   -
AtNCED1 : ------------------------------------------------------------------- :   -

VP14    : -------------------------------------------MQGLAPPTSVSIHRHLPARSRAR :  23
AtNCED2 : ----------------------------------------------------------MVSLLTMPM :   9
AtNCED3 : PGLYKPFTTLHRSQTNSLFSLLSSLLQEEKKQSLYTSQNRTYFNHQILIEHTKMASFTATAAVSGRW : 134
AtNCED5 : ------------------------------------------------MACSYILTPNPTKLNLSF :  18
AtNCED9 : ISGMYIYSLLSQDAHHSQYGQNTNLVLKKPIPKPQTAA----FNQESTMASTTLLPSTSTQFLDRTF :  69
AtNCED6 : ----------------------------------------------------------------MQH :   3
AtNCED4 : -----------------------------------------------------MDSVSSSSFLSSTF :  14
AtNCED1 : ------------------------------------------------------------------- :   -

VP14    : ASNSVRFSPRAVS-----SVPPAEC---LQAPFHKPVADLPAPSRKP---AAIAVPG---------- :  69
AtNCED2 : SGGIKTWPQAQID----LGFRPIK----RQPKVIKCTVQIDVTELTK--KRQLFTPRT--------- :  57
AtNCED3 : LGGNHTQPPLSSSQSSDLSYCSSLPMASRVTRKLNVSSALHTPPALHFPKQSSNSP----------- : 190
AtNCED5 : APSDLDAPSPSSS----VSFTNTK----PRRRKLSANSVSDTPNLLN--FPNYPSP----------- :  64
AtNCED9 : STSSSSSRPKLQS----LSFSSTL----RNKKLVVPCYVSSSVNKKSSVSSSLQSPTFKPPSWKKLC : 128
AtNCED6 : SLRSDLLPTKTSP-------RSHLLP---QPKNANISRRILINPFKIPTLPDLTSP----------- :  49
AtNCED4 : SLHHSLLRRRSSS--------PTLLR--INSAVVEERSPITNPSDNN---DRRNKPK---------- :  58
AtNCED1 : ------------------------------------------------------------------- :   -

VP14    : ---HAAAFRKAEGGKKQLNLFQRAAAAALDAFEEGFVANVLERPHGLPSTADPAVQIAGNFAPVG-E : 132
AtNCED2 : ---TATPFQHN---PLRLNIFQKAAAIAIDAAERALISHEQD--SPLPKTADPRVQIAGNYSPVP-E : 115
AtNCED3 : --AIVVKFKAKESNTKQMNLFQRAAAAALDAAEGFLVSHEKL--HPLPKTADPSVQIAGNFAPVN-E : 252
AtNCED5 : ---NPIIFEKD---TSRWNPLQRAASAALDFAETALLRRERS--KPLPKTVDPRHQISGNYAPVP-E : 122
AtNCED9 : NDVTNLIFKTTNQ-NPKLNPVQRTAAMVLDAVENAMISHERRR-HPHPKTADPAVQIAGNFFPVP-E : 192
AtNCED6 : ----VPSFVKLKPTYPNLNLLQKLAATMLDKIESSIVIPMEQN-RPLPKPTDPAVQLSGNFAPVN-E : 110
AtNCED4 : -TLHNRTNHTLVSSPPKLRPEMTLATALFTTVEDVINTFIDP---FSRPSVDPKHVLSDNFAPVLDE : 121
AtNCED1 : --MAEKLSDGSSIISVHPRPSKGFSSKLLDLLERLVVKLMHD-------ASLPLHYLSGNFAPIRDE :  58

VP14    : RFPVHELPVSGRIPPFIDGVYARNGANPCFDPVAGHHLFDGDGMVHALRIRNGAAESYACRFTETAR : 199
AtNCED2 : SSVRRNLTVEGTIPDCIDGVYIRNGANPMFEPTAGHHLFDGDGMVHAVKITNGS-ASYACRFTKTER : 181
AtNCED3 : QPVRRNLPVVGKLPDSIKGVYVRNGANPLHEPVTGHHFFDGDGMVHAVKFEHGS-ASYACRFTQTNR : 318
AtNCED5 : QSVKSSLSVDGKIPDCIDGVYLRNGANPLFEPVSGHHLFDGDGMVHAVKITNGD-ASYSCRFTETER : 188
AtNCED9 : KPVVHNLPVTGTVPECIQGVYVRNGANPLHKPVSGHHLFDGDGMVHAVRFDNGS-VSYACRFTETNR : 258
AtNCED6 : CPVQNGLEVVGQIPSCLKGVYIRNGANPMFPPLAGHHLFDGDGMIHAVSIGFDNQVSYSCRYTKTNR : 177
AtNCED4 : LFPTDCEIIHGTLPLSLNGAYIRNGPNPQFLPRGPYHLFDGDGMLHAIKIHNGK-ATLCSRYVKTYR : 187
AtNCED1 : TPPVKDLPVHGFLPECLNGEFVRVGPNPKFDAVAGYHWFDGDGMIHGVRIKDGK-ATYVSRYVKTSR : 124

VP14    : LRQERAIGRPVFPKAIGELHGHSG-IARLALFYARAACGLVDPSAGTGVANAGLVYFNGRLLAMSED : 265
AtNCED2 : LVQEKRLGRPVFPKAIGELHGHSG-IARLMLFYARGLCGLINNQNGVGVANAGLVYFNNRLLAMSED : 247
AtNCED3 : FVQERQLGRPVFPKAIGELHGHTG-IARLMLFYARAAAGIVDPAHGTGVANAGLVYFNGRLLAMSED : 384
AtNCED5 : LVQEKQLGSPIFPKAIGELHGHSG-IARLMLFYARGLFGLLNHKNGTGVANAGLVYFHDRLLAMSED : 254
AtNCED9 : LVQERECGRPVFPKAIGELHGHLG-IAKLMLFNTRGLFGLVDPTGGLGVANAGLVYFNGHLLAMSED : 324
AtNCED6 : LVQETALGRSVFPKPIGELHGHSG-LARLALFTARAGIGLVDGTRGMGVANAGVVFFNGRLLAMSED : 243
AtNCED4 : YNVEKQTGAPVMPNVFSGFNGVTASVAPGALTAARVLTGQYNPVNGIGLANTSLAFFSNRLFALGES : 254
AtNCED1 : LKQEEFFGAAKFMK-IGDLKGFFG-LLMVNVQQLRTKLKILDNTYGNGTANTALVYHHGKLLALQEA : 189
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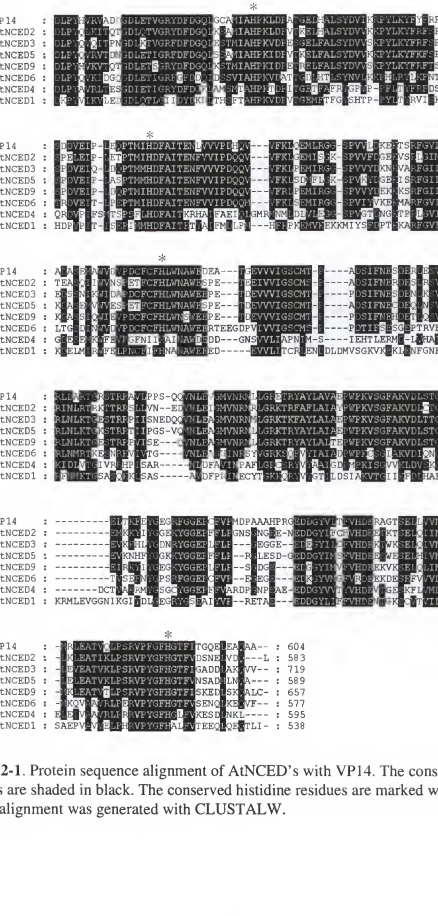

Fig. 2-1. Protein sequence alignment of AtNCED's with VP14. The conserved amino acids are shaded in black. The conserved histidine residues are marked with asterisks. The alignment was generated with CLUSTALW.

Cloning of *AtNCED4*

The *AtNCED4* was identified from the sequence of a 1.9 Mb of contiguous sequence from chromosome 4 of Arabidopsis thaliana (Bevan et al. 1998), the complete sequence of *AtNCED4* was within this 1.9 Mb region and the gene appears to be intronless. The AtNCED*4* encodes a protein of 594 amino acids and the protein shows 41% identity to VP14 (Fig. 2-1). The AtNCED4 protein contains a transit peptide and an AMP sequence at its N-terminal (refer to Fig. 3-2 in chapter 3). The cloning of *AtNCED4* into pGEM-3Z vector was described in materials and methods of chapter 3.

Cloning of *AtNCED5*

The *AtNCED5* was identified from the end sequence of a Arabidopsis BAC (bacterial artificial chromosome) clone T31A21. The deduced amino acid sequence of the ~ 400bp BAC end sequence showed significant sequence homology to VP14. The BAC clone T31A21 was obtained from Arabidopsis stock center and was sequenced from the end containing *AtNCED5*. The BAC clone contained full length *AtNCED5* and the gene was amplified by PCR (forward primer TCTCATGGCTTGTTCTTACATA, reverse primer TCTGGCTCTGCACTTTAAAC) and cloned into TOPO vector (Invitrogen). The AtNCED*5* also appears to be intronless and encodes a protein of 590 amino acid showing 65% identity to VP14 (Fig. 2-1). The AtNCED5 also has a transit peptide and an AMP sequence at its N-terminal (refer to Fig. 3-2 in chapter 3).

Sequence characteristics of *AtNCEDs*

As summarized in Fig. 2-2, *AtNCED2* and *AtNCED5* have about 65% sequence identity to VP14 at the amino acid level, and *AtNCED1* and *AtNCED4* have 40%

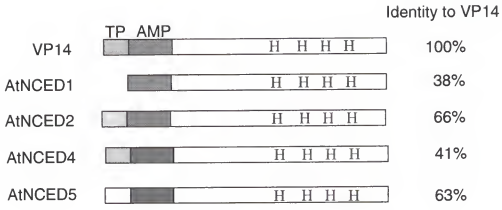

Fig. 2-2. Schematic diagram of structures of AtNCEDs and similarities to VP14. TP=transit peptide; AMP=a putative amphipathic α-helix; H=histidine residue. Four highly conserved histidine residues are found in AtNCEDs in a sequence alignment with VP14. These histidine residues are considered to chalate non-heme iron which is required for VP14 enzymatic function.

sequence identity to VP14 at amino acid level. *AtNCED2*, *AtNCED4* and *AtNCED5* are intronless, whereas *AtNCED1* contains multiple introns. Similar to VP14, AtNCED2, AtNCED4 and AtNCED5 proteins all have transit peptides resembling chloroplast localization signals, and they also have amphipathic helix regions at their N-terminals. AtNCED1 lacks transit peptide, though an amphipathic helix region (AMP) is conserved. In some other proteins, amphipathic sequences have been implicated in protein interactions and/or protein anchoring on membranes (Carr et al. 1991), and VP14 import assay showed its AMP sequence is required for anchoring VP14 to the thylakoid membranes (Tan et al., unpublished data). Alignment of AtNCEDs with VP14 shows striking homology at the C-terminus around four conserved histidine residues (Fig. 2-1). Conserved histidines are typical ligands of a non-heme iron cofactor in known

dioxygenases. Lignostilbene dioxygenase (LSD) and VP14 require non-heme iron for their activity (Kamoda et al. 1993, Schwartz et al. 1997).

***AtNCEDs* are Differentially Regulated by Water Stress**

The mRNA levels of the *AtNCED1, 2, 3, 4, 5, 9* genes were studied in detached leaves and roots in non-stressed and stressed conditions. The transcription of *Vp14* from maize tissues was also included for comparison. Poly(A)$^+$-RNA was used for the northern blots of *AtNCED2* and *Vp14* to enable to detect the expression, total RNA was used for all the other northern blots. Southern analyses were performed with individual gene probes under same stringency that was used for northern blots to make sure there is no cross hybridization between closely related genes (data not shown).

AtNCED3 and *AtNCED5* are both strongly induced by water stress in shoots of young seedlings, whereas *AtNCED1, 2,* and *9* are only weakly induced by water stress in seedlings. In contrast, *AtNCED4* is strongly down regulated by water stress in leaves. In detached roots, water stress has no effect on the expression of *AtNCED1, 3, 5*, and *9*, whereas *AtNCED2* is strongly inhibited by water stress. No expression of *AtNCED4* was detected in either roots or stressed roots (Fig. 2-3).

The *Vp14* gene of maize is strongly induced by water stress in leaves, and this expression pattern is similar to the expression of *AtNCED3* and *AtNCED5* in leaves. However, in roots *Vp14* expression is more similar to *AtNCED2*, where both show inhibition by water stress (Fig. 2-3).

The Carotenoid dioxygenase gene family in Arabidopsis

A search of the published Arabidopsis genome sequence with *Vp14* of maize revealed nine putative genes showing sequence homology to *Vp14*. The nine *AtNCEDs*

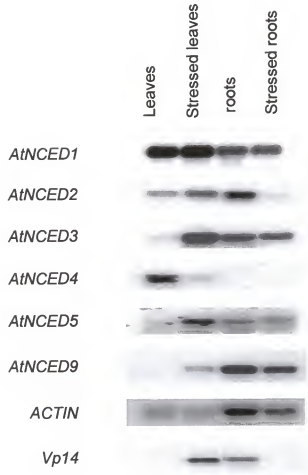

Fig. 2-3. Northern blots showing differential expression of *AtNCEDs* in normal and water stressed Arabidopsis seedings. Total RNA (20μg per lane) was used for all northern blots except *AtNCED2 and Vp14* in which poly(A)$^+$-RNA (2 μg per lane) was used.

show different degrees of homology to *Vp14* at the amino acid level (Table 2-1). Similar to *Vp14*, *AtNCED2, 3, 4 ,5, 6* and *9* appear to be intronless, while *AtNCED1, 7* and *8* contain multiple introns.

A distance tree was generated from AtNCEDs and its homologous proteins from different organisms. The predicted chloroplast transit peptides were taken off from the protein sequences of plants used in the tree. From the tree, *AtNCED2, 3, 5,* and *9* are grouped together with NCEDs from maize, tomato, bean, and avocado, defining a sub-group of genes that are more related to NCED than the other *AtNCEDs* (Fig. 2-4). Therefore, *AtNCED2, 3, 5* and *9* are very likely the NCED homologs and specifically function in the ABA biosynthesis. *AtNCED6* is more closely related to *Vp14* than *AtNCED1* and *AtNCED4*, hence it may still function as an NCED. AtNCED1 and PaNCED2 are grouped together since both lack the predicted chloroplast transit peptides. PaNCED2 was shown incapable of *in vitro* cleavage of 9-cis epoxycarotenoids, thus is not involved in the ABA biosynthesis (Chernys et al. 2000). Recombinant AtNCED1 could catalyze oxidative cleavage of lutein, zeaxanthin, violaxanthin, 9-cis violaxanthin, and 9-cis neoxanthin (Schwartz and Zeevaart, personal comm). *AtNCED7* and *AtNCED8* show weak sequence homology to *Vp14*, whereas they are more closely related to a β-carotene dioxygenase from *Drosophila melanogaster* (Dm VitA) and RPE65 protein from animals (Fig 2-4). The Drosophila β-carotene dioxygenase catalyzes the centric cleavage of β-carotene to produce two molecules of retinal in the vitamin A biosynthetic pathway (Lintig and Vogt. 2000). Although the biochemical function of RPE65 is unknown, RPE65 is specifically expressed in the retinal pigment epithelium of the eye, and mutations in this gene have been associated with retinitis in humans (Morimura et al.

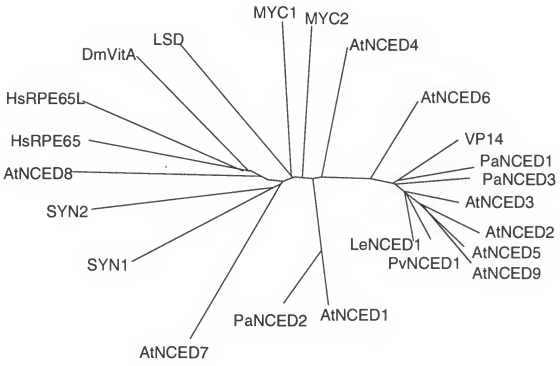

Fig. 2-4. A tree showing the evolutionary relationship of dioxygenase gene family. The tree was generated using CLUSTALW. Maize VP14, avocado PaNCED1, 2, 3, tomato LeNCED, bean PvNCED1, Drosophila DmVitA, animal RPE65, RPE65L, and bacteria MYC1, 2 , SYN1, 2, LSD are the dioxygenase family showing homology to AtNCED.

1998). Therefore, RPE65 is very likely the homolog of Drosophila β-carotene dioxygenase. The AtNCEDs also share sequence homology to lignostilbene dioxygenase (LSD) from *Pseudomonas paucimobilis*, and several proteins of unknown functions (SYN, MYN) from *Cyanobacterium synechocystis* (Fig. 2-4).

Table 2-1. General information of AtNCEDs

	Gene Accession #	Chromosome Location	Identity to VP14
AtNCED1	AJ005813	3	38%
AtNCED2	AL021710	4	66%
AtNCED3	AB028617	3	68%
AtNCED4	AL021687	4	41%
AtNCED5	AC074176	1	63%
AtNCED6	AB028621	3	56%
AtNCED7	AC007659	2	23%
AtNCED8	AL161582	4	24%
AtNCED9	AC013430	1	68%

DISCUSSION

Water stress induces endogenous ABA biosynthesis, and it has been demonstrated that transcriptional inhibitors can prevent ABA accumulation under water stress conditions, indicating that ABA biosynthesis is regulated at the transcriptional level (Guerrero and Mullet 1986). Biochemical evidence has shown that cleavage of epoxycarotenoids is the key regulatory step in ABA biosynthesis. Induction of NCED

gene expression by water stress has been observed for *Vp14* of maize, *LeNCED1* of tomato, *PvNCED1* of bean, and *VuNCED1* of cowpea (Tan et al. 1997, Burbidge et al. 1997, Qin et al. 1999, Iuchi et al. 2000). The *PvNCED1* of bean and *VuNCED1* of cowpea are strongly induced in water stressed plants and they are mainly responsible for ABA biosynthesis in water stressed condition.

It appears that there is a mixed and a balanced expression among *AtNCEDs* in leaves, roots, and in response to water stress. In *PvNCED1* of bean, a nice correlation was observed among *PvNCED1* mRNA, protein, and ABA levels in detached leaves and roots (Qin et al. 1999). We did not observe any obvious *AtNCED* increase at the transcriptional level in detached roots. However, we do not have a qualitative comparison of the transcription in non-stressed and stressed roots. Also, the detached roots are an artificial system that may not completely reflect the situation in vivo. Actually, the expression of *AtNCED2* and the *Vp14* of maize both show inhibition in detached roots. The same expression pattern indicates that these two genes might be orthologs, although *AtNCED2* does not show the same amount of induction as *Vp14* by water stress in leaves. In water stressed leaves, the expression of *Vp14* is more like *AtNCED3* and *AtNCED5*. It is possible that the regulation of the NCED ortholog changed since the divergence of maize and Arabidopsis. *Vp14* is also expressed in embryos. It would be interesting to find out which *AtNCEDs* are expressed in developing siliques.

CHAPTER 3
STUDY OF LOCALIZATION OF FOUR *9-CIS* EPOXYCAROTENOID DIOXYGENASE PROTEINS FROM ARABIDOPSIS

Abstract

Chloroplast membranes contain a diverse group of carotenoids. In addition, epoxycarotenoids, the ABA precursor, are localized in the membranes of plastids (Zeevaart and Creelman 1988, Watson and Li 1995). Carotenoids are hydrophobic molecules that are associated with membranes. Therefore, The carotenoid dioxygenase localization and membrane association are potential points of post-translational regulation. We studied protein localization of AtNCED1, 2, 4 and 5 using an *in vitro* chloroplast import assay. AtNCED2 and 5 show significant sequence homology to VP14, thus are very likely to function in the ABA biosynthesis, whereas AtNCED1 and 4 may use other carotenoids as substrates. The results showed that AtNCED2, 4, and 5 proteins are differentially localized within chloroplasts. AtNCED2 is partially localized in the stroma, and partially associated with the thylakoid membranes. In contrast, AtNCED4 is localized only in the stroma, whereas AtNCED5 is associated exclusively with the thylakoid membranes. The imported mature AtNCED5 is larger than its translation precursor suggesting modification post-import. The AtNCED1 lacks a putative transit peptide and it is not imported into chloroplasts. The differential localization of AtNCED proteins suggests that the post-translational regulation may be significant in regulating the substrate access, thus protein activity.

Introduction

It was proposed that the initial steps of ABA biosynthesis occur within plastids because the precursors of carotenoids are localized exclusively in the membranes of chloroplasts and other plastids (Zeevaart and Creelman 1988, Watson and Li 1995) (Fig. 3-1). It was shown that the zeaxanthin epoxidase of tobacco and the VP14 of maize are localized within chloroplasts (Marin et al. 1996, Tan et al. unpublished data). The final two steps in conversion of xanthoxin to ABA occur in the cytosol (Zeevaart and Creelman 1988, Watson and Li 1995).

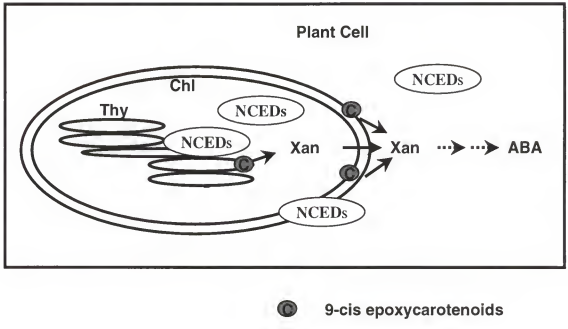

Fig 3-1. Possible localizations of NCED in a plant cell. The substrates 9-cis epoxycarotenoids of NCED are localized exclusively in the membranes of chloroplasts, including outer, inner envelope membranes and thylakoid membranes. The last two steps of ABA biosynthesis that converts xanthoxin to ABA occur in the cytosol. Chl, chloroplast; Thy, thylakoids; Xan, xanthoxin.

Recombinant VP14 expressed in *E. coli* is water soluble (Schwartz et al. 1997). To study the mechanism by which water soluble VP14 accesses hydrophobic membrane localized substrates, VP14 localization and membrane association were studied (Tan et al. unpublished data). VP14 contains a transit peptide and a putative amphipathic α-helix (AMP) sequence at its N-terminus. In some other proteins, amphipathic helices have been implicated in protein interactions and/or protein anchoring on membranes (Carr et al. 1991). Chloroplast subfractionation after import assays showed that VP14 partially exists soluble in the stroma fraction and partially associates with the thylakoid membranes. Functional analysis experiments showed that AMP was required but not sufficient for the association of VP14 to the thylakoid membranes. Deletion or destruction of the AMP abolished the association of VP14 to the thylakoids. However, a chimeric protein containing the transit peptide and the AMP sequence of VP14 fused with glutathione S-transferase (GST) was not sufficient to target GST to thylakoids, though GST was localized to the stroma. Analyses of a series of truncation mutants showed that AMP plus ~ 160 N-terminal amino acid sequence of mature VP14 is required to target the GST to thylakoids. Washing treatments and competition experiments demonstrated that the association of VP14 to the thylakoids has specificity. VP14 remained bound to the thylakoid following extensive washing with chaotropic reagents such as 6M urea or 0.1M Na_2CO_3. A competition experiment which using cold Vp14 to compete thylakoid bound hot VP14 showed that cold VP14 competes off the thylakoid bound VP14 in a concentration dependent manner, suggesting that VP14 interacts with the thylakoid through a specific partner or partners in the membranes.

In this study, we determined whether four VP14-like proteins from Arabidopsis, AtNCED 1, 2, 4, and 5, could be imported into chloroplasts and if so, in which compartment they are localized.

Materials and Methods

Plasmid Constructs

The Sph1 restriction site (GCATGC) in the pGEM-3Z vector (Promega) was destroyed by digesting the vector with Sph1 restriction enzyme, then blunted with Klenow fragment, and self-ligated. The purpose of destroying the Sph1 site is to avoid the confusion of the Sph1 internal ATG and the gene start codons. The AtNCED1, 2, 4, and 5 were then subcloned into this pGEM-3Z vector under the Sp6 promoter.

AtNCED1: a construct containing the full length *AtNCED1* cDNA sequence was digested with SalI and SmaI, and the insert was cloned into pGEM-3Z vector under control of the Sp6 promoter. The construct was linearized with EcoRI at the 3' end to make the transcription template.

AtNCED2: a construct containing the full length *AtNCED2* genomic sequence (no introns) was digested with Xho1 and SmaI, and ligated into SalI and SmaI sites of PGEM-3Z vector (Xho1 and SalI have compatible cohesive ends). The construct was linearized with EcoRI to make the transcription template.

AtNCED4: The primers for cloning were designed based on the published sequence. The forward primer: 5' CAAAAGCTGCAG**AATG**GACTCTGTTTCTTCTTCTT 3', and the reverse primer CAACAATCTAGAGCGATTTGTTGTACGGGACG, include a Pst1 site and a Xba1 site (underlined) respectively, to facilitate cloning. The ATG (bold) in the forward primer

is the AtNCED4 start codon. The PCR fragment amplified from Arabidopsis genomic DNA as template was digested with Pst1 and Xba1, and cloned into PGEM3Z. The construct was linearized with EcoRI to make the transcription template.

AtNCED5: The full length *AtNCED5* sequence was amplified by PCR using BAC clone T31A21 as template. (Forward primer: TCTC**ATG**GCTTGTTCTTACATA, reverse primer: TCTGGCTCTGCACTTTAAAC). The PCR fragment was cloned into TOPO vector (Invitrogen Inc), and the resulting plasmid digested with Hind III and Xba1. The insert was cloned into PGEM3Z vector for import assays. The template was linearized with XbaI.

In vitro Transcription and Translation

In vitro transcription and translation were performed according to Cline et al. (1993). The transcription reaction contains 5 mM DTT, 50units/ml RNasin (Promega), 0.5 mM NTPs, 50 µg linearized template/ml, 500units/ml SP6 DNA polymerase in 1× SP6 DNA polymerase buffer (Promega). The reaction was incubated at 40°C for 60 min. The synthesized messenger RNA was precipitated with ethanol and quantitated spectrophotometrically. A 50µl translation reaction contained 30µl premade wheat germ cell-free extract, 5 µl premix of all amino acids except leucine, 5µl 5 × buffer, 5 µl (40 ng) *in vitro* synthesized messenger RNA, 5 µl 3^{H}-leucine (3000 ci/mol, Dupont). The reaction was incubated at 25°C for 1 hour and stopped by putting tubes in ice. Translation product was diluted with equal amount of 60mM leucine in 2 × import buffer (IB, 50mM hepes/KOH pH 8 and 0.33 M sorbitol) immediately before the import assay.

Import Assay

The protein import assay and the subfractionation of chloroplasts were performed according to Cline et al. (1993). The import reactions were carried out in 1× IB containing 5 mM Mg-ATP at 25°C under light. The unimported proteins were removed by washing with IB and treatment with thermolysin. The chloroplasts were then purified by centrifugation onto a 35% percol cushion, and rinsed with IB. The chloroplasts were lysed by a hypertonic solution containing 20 mM EDTA. For subfractionation, chloroplasts were lysed in 10 mM Hepes-KOH (pH 8.0) containing 10 mM $MgCl_2$ for 5 to 10 min on ice. Unlysed chloroplasts were separated through percol cushion centrifugation. The lysed chloroplasts were collected by centrifugation at 4,200 rpm for 8 min. The pellets that contained thylakoids were rinsed twice with IB and were suspended in SDS for proteins. The supernatant fraction was further centrifuged at 18,000g for 2 hrs to separate the envelope membranes and stroma. The thylakoids, envelope membranes, and stroma were lysed in 20 mM EDTA and denatured in 15% SDS in boiling water for 5 min, and analyzed in 12% SDS-PAGE. The gels were treated with DMSO and PPO/POPOP and dried before radioactive proteins were detected by fluorography. The rubisco small subunit (RSB) and the light harvest chlorophyll protein (LHCP) were used as controls in chloroplast import and subfractionation experiments

Note: Part of the data in this chapter was contributed by Bao-Cai Tan. I made all the constructs and did the *in vitro* transcription experiments, and he performed all the *in vitro* translation and import assays.

Results

N-terminal Sequence Comparison of AtNCEDs with VP14

Similar to VP14, AtNCED2, 4, and 5 appear to have N-terminal extensions that function as transit peptides to direct transport of the cytoplasm synthesized protein into chloroplasts. AtNCED1 lacks an evident transit peptide (Fig. 3-2A). It has been suggested that high content of serine and threonine and lack of acidic residues are the essential features of targeting nuclear-encoded proteins into chloroplasts (Cline and Henry 1996). The AtNCED4 and AtNCED5 are both enriched in serine and threonine at their N-terminal sequence, whereas AtNCED2 is not so enriched.

The VP14 has a putative amphipathic α-helix at the N-terminus. Analysis of this region with PHD program (www.public.iastate.edu) revealed a secondary structure of a strong probability of forming α-helix (Tan et al. unpublished data). AtNCED1, 2, 4, 5 all have regions of about 20 amino acids at their N-terminals that have potential to form an α-helix (Fig. 3-2A). The helical wheel plots of VP14, AtNCED1, 2, 4, 5 show strong characteristics of amphipathy (Fig. 3-2B). One side of the helices is enriched in charged or hydroxyl residues, while the other sides contain only neutral amino acids.

To test the localization of AtNCED proteins, the *AtNCED* genes were *in vitro* transcribed and translated, and the translated proteins were incubated with pea chloroplasts. The chloroplasts were then treated with thermolysin to degrade proteins that were located outside of chloroplasts. The chloroplasts were then subfractionated into envelope, stroma, and thylakoids fractions.

A

```
VP14      MQGLAPPTSVSIHRHLPARSRARASNSVRFSPRAVSSVPPAECLQAPFHKPVADLPAPSRKP
AtNCED1
AtNCED2                 MVSLLTMPMSGGIKTWPQAQIDLGFRPIKRQPKVIKCTVQIDVTELT
AtNCED4               MDSVSSSSFLSSTFSLHHSLLRRRSSSPTLLRINSAVVEERSPITNPSDNNDR
AtNCED5             MACSYILTPNPTKLNLSFAPSDLDAPSPSSSVSFTNTKPRRRKLSANSVSDTPN

VP14      AAIAVPGHAAAPRKAEGGKKQLNLFQRAAAAALDAFEEGFVANVLERPHGLPSTADPAVQIA
AtNCED1         MAEKLSDGSSIISVHPRPSKGFSSKLLDLLERLVVKLMHDASLPLHYLSGNFAPIR
AtNCED2   KKRQLFTPRTTATPPQHNPLRLNIFQKAAAIAIDAAERALISHEQDSPLPKTADPRVQIAGN
AtNCED4   RNKPKTLHNRTNHTLVSSPPKLRPEMTLATALFTTVEDVINTFIDPPSRPSVDPKHVLSDNF
AtNCED5   LLNFPNYPSPNPIIPEKDTSRWNPLQRAASAALDFAETLLLRRERSKPLPKTVDPRHQISGN
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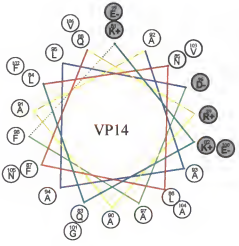
B



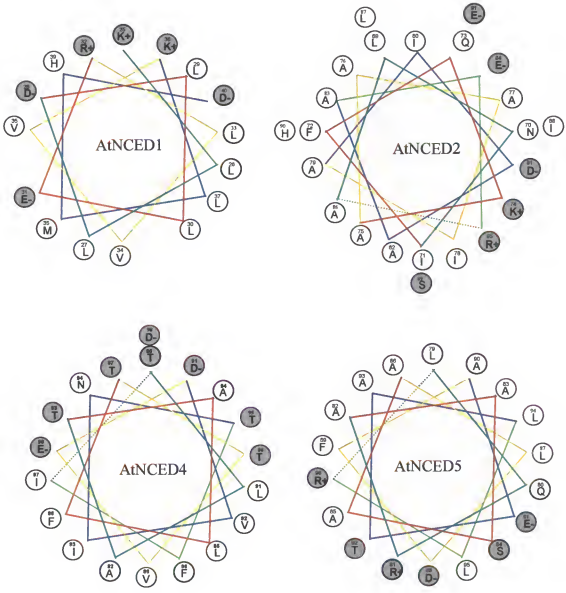


Fig. 3-2. (A). N-terminal sequence alignment of VP14 and AtNCED1, 2, 4, 5 showing the transit peptides and the amphipathic helix sequences (AMP). AMP sequences are underlined. (B). The amphipathic helix wheel plots of the AMP sequences.

AtNCED1 is Not Imported into Chloroplasts

Consistent with not having a transit peptide, the *in vitro* translated AtNCED1 protein was not targeted into chloroplasts (Fig. 3-3A). The *in vitro* translated AtNCED1 showed multiple bands on SDS-PAGE that may due to degradation. The AtNCED1 protein was digested away in chloroplasts treated with thermolysin indicating that AtNCED1 is not imported.

AtNCED2 Exists in Both Stroma and Thylakoid Fractions

The AtNCED2 is targeted into chloroplasts (Fig 3-3A). Further chloroplast fractionation showed that AtNCED2 exists in both stroma and thylakoid fractions. The majority of the protein is soluble in stroma with a lessor fraction bound to thylakoid membranes (Fig. 3-3B). The size of protein in thylakoids is smaller than its translated precursor suggesting a cleavage of the transit peptide. However, the stroma form migrates faster than the thylakoid bound form suggesting partial degradation or modification. The localization pattern of AtNCED2 resembles VP14 that is also localized in both stroma and thylakoids.

AtNCED4 is Localized Only in the Stroma Fraction

The AtNCED4 is imported into chloroplasts. The size of the imported protein is smaller than its translated precursor indicating a cleavage of the transit peptide (Fig. 3-3A). The imported AtNCED4 is localized exclusively in the stroma fraction (Fig. 3-3B).

AtNCED5 is exclusively associated with the thylakoids

The apparent size of the imported mature protein of AtNCED5 as shown in SDS-PAGE is interestingly larger than its translation precursor (Fig. 3-3A). One possibility of

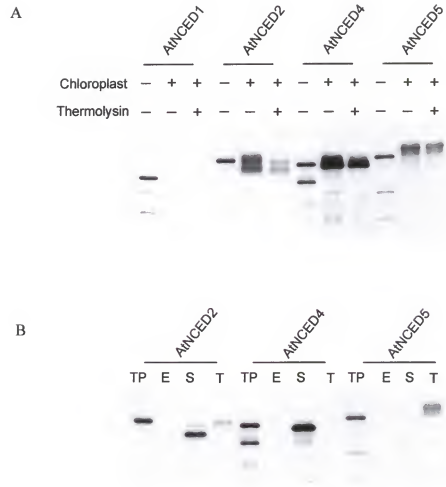


Fig. 3-3. *In vitro* chloroplast import assay of AtNCEDs. AtNCEDs were in *vitro* translated in the presence of ^{3}H-leucine and incubated with fresh isolated pea chloroplasts under the import conditions (Cline et al. 1993). A. Fluorography of SDS-PAGE on the AtNCEDs from imported chloroplasts before and after treated with thermolysin. B. Subfractionation of the imported chloroplasts into three fractions, envelop (E), stroma (S), and thylakoid (T). Translational precursor is labeled as TP. All samples were loaded on a per chloroplast basis.

this size shift may be resulted from post-import modification, such as phosphorylation. In contrast to AtNCED4, AtNCED5 is exclusively associated with the thylakoid membranes (Fig 3-3B).

Discussion

Carotenoids are localized in the membranes of plastids, and they are hydrophobic molecules. The sub-cellular localization and membrane association of the carotenoid dioxygenase are potential points of post-translational regulation. AtNCED proteins display a striking difference in localization indicating that post-translational regulation may be significant. Study of the sequence that is required for VP14 membrane association demonstrated that the AMP plus ~160 N-terminal amino acids of mature VP14 is required for targeting VP14 to the thylakoids (Tan et al., unpublished data). AtNCED1, 2, 4, and 5 all have AMP sequences. The AMP and the ~160 amino acid sequence downstream of the AMP is highly conserved between AtNCED2 and VP14 which may explain the similar localization pattern. These two proteins are localized in both the stroma and the thylakoid fractions. The stroma localized form and the thylakoid bound form may reflect two states of enzyme activity, the stromal form is inactive, while the thylakoid bound form is active. The interconversion between these two forms may be a point of enzymatic regulation. The similar localization of AtNCED2 and VP14 further supports the possibility that these two genes are orthologs. Different from AtNCED2, AtNCED5 is exclusively bound to the thylakoid membrane. This localization pattern is similar to the PvNCED1 of bean (Qin et al. 1999). However, the size of the imported mature AtNCED5 appears larger than its precursor suggesting that the protein is modified post-import. The AMP sequence of AtNCED5 contains a serine and a threonine that

could be phosphorylated after AtNCED5 is imported into chloroplasts, resulting in an apparent size shift of the mature protein. In contrast to AtNCED5, AtNCED4 is only localized in the stromal fraction. The AMP sequence of AtNCED4 and the sequence further downstream are divergent from VP14. Domain swapping experiments would help us to determine the sequence involved in the differential localization and post-import modification. AtNCED1 is not localized into chloroplasts. However, the outer envelope membranes of plastids contain the carotenoid substrates that AtNCED1 was shown to cleave. Perhaps the cytoplasm localized AtNCED1 can access the substrates from outside of the chloroplasts.

CHAPTER 4
CHARATERIZATION OF *AtNCED2* TRANSGENIC PLANTS AND SCREENING OF *AtNCED* KNOCKOUT MUTANTS

Abstract

Considerable progress has been made in determining the function of ABA in regulating seed development and responses to various environmental stresses. However, little is known about the function of ABA in normal plant growth and development. We studied the functions of ABA and the AtNCED gene family by constructing AtNCED transgenic plants and screening AtNCED knockout mutants. The *AtNCED2* gene shows high sequence homology to the *Vp14* of maize, thus is very likely to function as an NCED in Arabidopsis. The transgenic plants of *AtNCED2* promoter-GUS, over-expression, and antisense were generated and analyzed. The *AtNCED2* promoter-GUS transgenic lines showed GUS staining in the pericycle and cortex cells surrounding lateral root initials. IAA treatment that induced lateral root formation also strongly induced GUS staining in these cells, suggesting that ABA may play a role in the lateral root formation. The GUS staining was also observed in a narrow ring of cells in root tips, in the shoot apical meristem, in the flower primordia, and in the abscission zone of mature flowers. Transgenic plants over-expressing AtNCED2 show delayed cotyledon and leaf growth, delayed flowering, and inhibited lateral root growth. The transgenic plants also exhibited male sterility and anthocyanin pigment accumulation in the petioles and sepals of flowers. Knockout mutants were screened for *AtNCED2, 3, 4, 5, 6* and *9* using the

Knockout Facility at the University of Wisconsin. Two mutants were identified each for *AtNCED2* and *AtNCED5*, and candidate mutants have been identified for all the others, and they are currently undergoing further screening.

Introduction

Abscisic acid (ABA) regulates seed development and dormancy, and gene expression in response to environmental stresses such as drought, salinity, and cold. The ABA is a sesquiterpenoid (C_{15}), and it was proposed that ABA is synthesized from oxidative cleavage of epoxycarotenoids in higher plants (Zeevaart et al. 1989, Parry et al. 1992a, Walton and Li 1995). Direct evidence for the oxidative cleavage reaction came from the isolation of the *vp14* mutant of maize and cloning of the corresponding gene (Tan et al. 1997). Enzyme assays showed that the recombinant VP14 protein specifically catalyzes the oxidative cleavage of *9-cis* epoxycarotenoids, giving rise to 2-cis, 4-trans-xanthoxin, the immediate precursor of ABA, and C25-apocarotenoids (Schwartz et al. 1997). This step is the first committed and presumably the key regulatory step of ABA biosynthesis. Previous evidence indicated that the conversion of xanthoxin to ABA is constitutive and is not up-regulated by water stress, thus it is not rate limiting (Sindhu and Walton 1987). The substrate carotenoids are abundantly available at high levels relative to ABA. Therefore, formation of these epoxycarotenoids is unlikely to be the key regulated steps in regulating the ABA biosynthesis. The key regulatory enzyme catalyzing the oxidative cleavage of *9-cis* epoxycarotenoids, *9-cis* epoxycarotenoid dioxygenase (NCED), is encoded by a complex gene family (Tan et al. 1997, Qin and Zeevaart 1999).

ABA-deficient mutants that are impaired at a number of different steps of ABA biosynthetic pathway have been isolated from several species (reviewed by Taylor 1991). *Vp14* of maize and *notabilis* of tomato are the known mutants that were impaired in the oxidative cleavage step. The *vp14* of maize was identified as a *viviparous* mutant in which the embryos start to germinate while still attached to the mother ear. The seedlings of *vp14* show no discernible phenotype either in greenhouse or field growth conditions. The mutant has reduced levels of ABA in embryo and water-stressed leaves (Tan et al. 1997). The *Notabilis* of tomato has a very mild wilty plant phenotype (Taylor et al. 1988). In Arabidopsis, mutants impaired at both the upstream and downstream of the oxidation cleavage step were identified. *Aba1* was isolated in a screen for revertants of non-germinating gibberellin-sensitive mutants (Koornneef et al. 1982). The *aba1* mutant is impaired in the epoxidation of zeaxanthin to violaxanthin (Rock and Zeevaart 1991). The *aba2* and *aba3* mutants were isolated from a screen for seeds that were able to germinate in the presence of a gibberellin biosynthesis inhibitor paclobutrazol. These two mutants have reduced seed dormancy and increased rates of water loss (Leon-Kloosterziel et al. 1996). Biochemical characterization showed that *aba2* is blocked in the conversion of xanthoxin to ABA-aldehyde, and *aba3* is blocked in the last step of ABA biosynthesis, the conversion of ABA-aldehyde to ABA (Schwartz et al. 1997). However, despite the efforts from several labs, mutants in the epoxycarotenoid cleavage step have not been isolated from Arabidopsis presumably due to substantial genetic redundancy. Based on available Arabidopsis genomic sequence, there are at least four genes of Arabidopsis (*AtNCED2, 3, 5,* and *9*) that are very closely related to the *NCED* genes from

maize, bean, and tomato, and there are three more genes (*AtNCED1, 4* and *6*) that also show significant but more distant homology to *Vp14* of maize and *notabolis* of tomato.

We took advantage of the availability of T-DNA insertion collections from the *Arabidopsis* knockout facility at University of Wisconsin (http://www.biotech.wisc.edu/arabidopsis/) and screened knockout mutants for *AtNCED2, 3, 4, 5, 6,* and *9* (Refer to Fig. 2-4) using gene specific primers. Two knockout mutants have been identified each for *AtNCED2* and *AtNCED5*, and candidates have been identified for all the others, and further screenings are in progress. We also studied the functions of *AtNCED2* by constructing and characterizing of promoter-GUS, over-expression, and antisense transgenic plants.

Materials and Methods

Constructs

35S-AtNCED2 construct: Full length *AtNCED2* genomic DNA (*AtNCED2* is intronless) including 120 bp 5' the untranslated region was cloned into the pBI 121 vector between BamH1 and Sst1 sites under the control of 35S promoter.

AtNED2 promoter-GUS construct: a 2.3 kb promoter region of *AtNCED2* was amplified by PCR from a construct containing the promoter and partial coding region of *AtNCED2*. The PCR product was amplified using T7 as forward primer, and the sequence of reverse primer is : CGTGGATCCCATGGCTTTTGTTTTCTTATC. The PCR fragment was digested with HindIII and BamHI, cloned into pBluescript, and then further cloned into the pBI101 vector fused in-frame in front of GUS. The final construct was sequenced.

Seed Handling and Plant Transformation

Arabidopsis seeds were sterilized in 50% bleach solution for 5 min, rinsed three times with sterilized water, and cold treated in ice for 3-5 days before germinating on GM plates. GM plate contains 1× MS (Murashige and Skoog basal medium), 1% sucrose, 0.2% phytogel, pH 5.7. For Kanamycin selection, 50 μg/mg kanamycin was included in the medium. Transformation was performed according to Clough et al (1998).

Screening of AtNCED Knockout Mutants

The mutant screening procedure is described in details on the webpage of knockout Facility at University of Wisconsin: http://www.biotech.wisc.edu/arabidopsis/. In brief, the knockout Facility has a collection of 60,480 Arabidopsis (ecotype WS) lines that have been transformed with the T-DNA vector pD991. We designed *AtNCED2* and *AtNCED5* gene-specific primers from both 5' and 3' ends. The Facility starts the first round of PCR (polymerase chain reaction) on 30 super DNA pools, each pool contains DNA from 2025 T-DNA transgenic lines, using T-DNA left border primer (JL202) combined with either 5' or 3' of the gene specific primer. The PCR reactions were resolved on 1.2% agarose gels and blotted on nylon membranes for Southern analysis. The hybridized bands were subcloned into TOPO vector (Invitrogen) and sequenced. If a hit is found in the first round of PCR which narrows down the hit to 2025 lines, then a second round PCR is performed on 9 DNA pools, each pool contains DNA from 225 lines which narrows down the hit to one particular pool of 225. Seed pools of these 225 lines that organized in a form of 25 seed pools each containing 9 lines can be ordered from Arabidopsis Stock Center. Half amount of seeds from each tube were sterilized and germinated. DNA was extracted from the young seedlings. PCR and Southern analysis

were performed to narrow down the hit to 9 lines. Last of all, the rest half amount of seeds were grown up from the tube that the hit was found, and DNA was extracted from those individual plants and the hit was narrowed down to a final plant.

The primers used for screening *AtNCED2* mutant are:

5' primer: CCTTCTTCTTTTTCTCTTCTCATCGAACT

3' primer: AAATGTGCCATGAAACCCATACGGTACTC

The primers used for screening *AtNCED5* mutants are:

5' primer: ATCAAGTTATCGTATGTTCCGTGTCTATT

3' primer: ACAATATGAAGCTCCGATTCCCAACTCTC

The primers used for screening *AtNCED4* mutants are:

5' primer: TAGTGGCTCAAAGAAACCAGGTCGGAGAA

3' primer: TTTAGCGTCCATCACCAGAAACTTCGATT

The primers used for screening *AtNCED6* mutants are:

5' primer: GCTCAAACCAACGTATCCAAACTTAAACC

3' primer: AAACGTAAACCTAAAAAGAGGAGATAGCA

The primers used for screening *AtNCED4* mutants are:

5' primer: GAAACAGAAGAAGCCACAAAAAAAGACAA

3' primer: AAACCGCACCCCAAAAGAAACAACAAACA

The primers used for screening *AtNCED9* mutants are:

5' primer: GATAAATTGTGGGAGATAAACAAGATGAG

3' primer: CTCAGGCAACAATCCTATGTCACTAATCA

Results

AtNCED2 Promoter-GUS Transgenic Plants Show Localized GUS Staining Pattern

To study the *AtNCED2* gene expression at the cellular level, a construct containing 2.3 kb *AtNCED2* promoter fused to beta-glucuronidase reporter gene (GUS) was introduced into Columbia wildtype plants. The transgenic plants showed a striking localized GUS staining pattern. In well-watered seedlings, GUS staining was observed in the pericycle and cortex cells surrounding new lateral root initials (Fig. 4-1,C). Pericycle cells are a single layer of cells that surround vascular tissue, and they are organized longitudinally along the length of the roots. Lateral roots are initiated from pericycle cells that are located adjacent to a xylem pole, thus there are two column of lateral roots 180 degree apart (Blakely et al. 1982). The GUS staining apparently precedes the lateral root initials and it seems to predict the position of new lateral roots (Fig. 4-1, C). IAA treatment that induces lateral root formation also strongly induces GUS staining (Fig 4-1, D). The staining pattern suggests that ABA may have a function in lateral root formation. Gus staining was also observed in the base of mature lateral roots (Fig 4-1, B), and in a narrow ring of cells in root caps (Fig 4-1, A). In shoots, GUS staining was detected in the apical shoot meristem that is known to contain high levels of ABA (Fig. 4-1, E). GUS staining was also detected in the flower primordia. The staining was restricted to the abscission zone in mature flowers (Fig 4-1, F). In water stressed seedlings, GUS expression is induced in guard cells in the petioles and in the senescent leaves near the base of rosette leaves (Fig 4-1, G and H).

The above staining patterns were consistently observed in more than 20 independent transgenic lines. Southern blots showed that most transgenic lines contain low copy number of transgene. Several lines showed that intense GUS staining in the

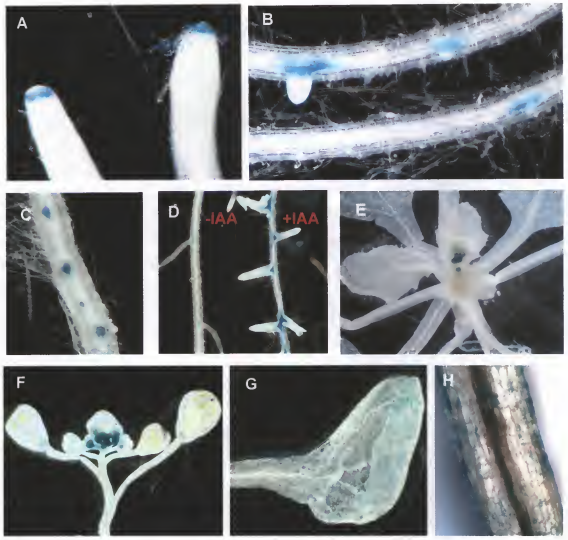


Fig. 4-1. *AtNCED2* promoter-GUS expression in transgenic plants. Expression (A) in a narrow ring of cells in root tips. (B) in the base of mature lateral roots. (C) in the pericycle and cortex cells surrounding new lateral initials. (D) is induced by IAA in the base of mature lateral roots. (E) the apical shoot meristem. (F) the primordia of young flowers and in the abscision zone of mature flowers. (G) and (H) is induced in the stomatal cells in senescent leave and in the petiole. A-F are expressions in well watered seedlings, G and H are from water stressed seedlings.

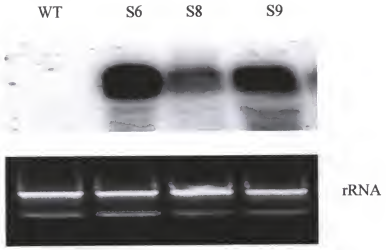


Fig. 4-2. Northern blot of AtNCED2 RNA expression in columbia, and transgenic lines S6, S8, and S9. Bottom panel is rRNA on agarose as loading control.

stems and in the petioles of the shoots, and the intense staining is correlated with high copy number of tandem insertions (data not shown).

Characterization of 35S-AtNCED2 Transgenic Plants

Generating transgenic plants showing high level of transgene expression

The *AtNCED2* gene under the control of the cauliflower mosaic virus 35S promoter was introduced into Columbia wildtype plants. Three transgenic lines, S6, S8, and S10, were chosen from 15 independent transgenic lines because they show higher levels of AtNCED2 RNA expression compared to wildtype based on northern analysis (Fig. 4-2). Plants homozygous for the transgene were obtained by self-pollinating each transgenic line. The expression of the transgene in the homozygous progeny was analyzed by northern blots. S6, S8, and S9 all express much higher level of *AtNCED2* RNA than wildtype plants (Fig. 4-2).

The bulk ABA levels in the transgenic lines were measured in non-stressed and stressed conditions. In non-stressed tissues, S6 has twice as much ABA as in the wildtype, while S8 and S9 do not show any increases in the ABA content. In stressed tissues, S6 has same level of ABA as in the wildtype, while S9 and S8 have slightly decreased level of ABA (Table 4-1).

Table 4-1. Characterization of AtNCED2 overexpression transgenic plants

	Columbia	S6	S9	S8
ABA content (NS)[A]	0.37	0.72	0.32	0.43
ABA content (S)[A]	2.10	2.19	1.44	1.88
Rosette leaf [B]	12 ± 2.5	18 ± 3.1	16 ± 2.7	14 ± 2.3
Lateral root[C]	1.9 ± 0.65	1.0 ± 0.64	1.2 ± 0.49	1.56 ± 0.52
Sterility [D]	0	70%	44%	28%

[A] The unit of ABA content is μg/g dry weight; NS, non-stressed; S, stressed.
[B] Numbers of rosette leaf when the first inflorescence emerges. Numbers are from an average of 10 individual plants of each line.
[C] Numbers of lateral roots per centimeter of primary root length when primary root growth was normalized to about equal length. Numbers are from an average of 10 individual plants of each line.
[D] Percentage of sterile plants from 16 individual plants of each line.

Inhibited plant growth and development

The AtNCED2 overexpression transgenic plants exhibited several unique phenotypes. One of these is inhibited plant growth and development. All three transgenic seedlings show delayed cotyledon and true leave emergence, and root growth is slightly inhibited (Fig. 4-3A). The transgenic plants also exhibit delayed rosette leaf growth,

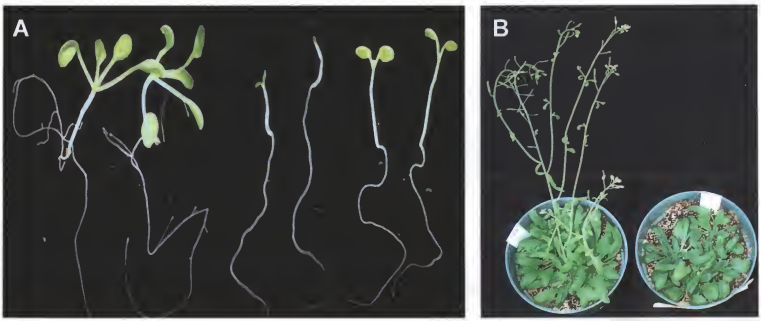


Fig. 4-3. (A) S6 and S9 seedlings show inhibited cotyleton emergence, true leaf and root growth. (B) transgenic plants show delayed leave growth and flowering.

shoot emergence, and flowering (Fig. 4-3B). Under same growth conditions, Columbia plants have an average of 12 rosette leaves when the first inflorescence emerges, while S6, S9, and S8 plants have 18, 16, and 14 rosette leaves, respectively (Table. 4-1). The severity of the phenotype is correlated with the *AtNCED2* RNA expression level, in which S6 is the most affected, and S8 is the least affected. Transgenic seeds do not show an obvious phenotype in germination or dormancy. When germination was scored three days after putting seeds on plates, seeds of both wildtype and transgenic lines were fully germinated, though the radicles of wildtype were significantly longer than that of transgenic lines (data not shown).

Inhibited lateral root growth

Lateral root growth is inhibited in transgenic lines. Transgenic plants have fewer and shorter lateral roots (Fig. 4-4, C). When the root growth was normalized to about equal length, Columbia plants had an average of 1.9 lateral roots per centimeter of root length, while S6, S9, and S8 plants had 0.8, 1.2, and 1.6 lateral roots per centimeter of root length, respectively (Table 4-1). The lateral root phenotype is consistent with the promoter-GUS staining pattern in the pericycle cells that surround lateral root initials, and the promoter can be induced by IAA treatment. The data suggests a new hypothesis that ABA might play a role in lateral root initiation.

Male sterility

Transgenic plants also show high frequency of male sterility, in which the stamens contain less pollen or no pollen (Fig 4-4, B). Columbia plants grown in the same conditions had a normal flower and silique development, while there was always a

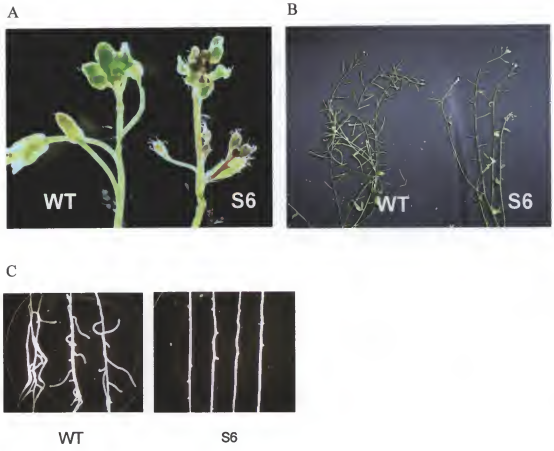


Fig. 4-4. 35S-AtNCED2 transgenic plants exhibit multiple plant phenotypes. A. Pigment is accumulated along the petiole and in the sepals of flowers. B. transgenic plants show male sterility. C. Lateral root growth is inhibited.

percentage of transgenic lines showing sterility (70%, 44%, and 28% for S6, S9, and S8, respectively) (Table 4-1). In the three transgenic lines, the most severely affected plants almost had no silique development, and the least affected plants only showed sterility in some branches. S6 plants showed higher frequency of severe sterility than S9 and S8 plants.

Pigmentation accumulation

Transgenic lines also displayed anthocyanin pigment accumulation on the petioles and sepals of flowers (Fig. 4-4A), and on the surface of siliques, while no pigmentation was observed in wildtype plants growing in the same conditions. The extent of pigmentation was correlated with sterility. Interestingly, this pigmentation phenotype mimics the wildtype plants grown in the stressed conditions such as strong light or drought.

Screening of *AtNCED* Knockout Mutants

To pursue the biochemical and physiological functions of each gene family member, we screened the 60, 000 Arabidopsis T-DNA insertion lines from Knockout Facility at the University of Wisconsin for all the genes showing homology to *NCED*, including *AtNCED 2, 3, 4, 5, 6* and *9*. Two knockout mutants were found for *AtNCED2*, one has an insertion 600 bp downstream of ATG and the other one has an insertion 900 bp downstream of ATG (Fig. 4-5). Two mutants were found for *AtNCED5*, one has an insertion 40 bp upstream of ATG, and the other one has an insertion 550 bp upstream of ATG and this insertion may still within the *AtNCED5* promoter region and thus may affect the initiation of transcription (Fig. 4-5). All above mutants have been narrowed

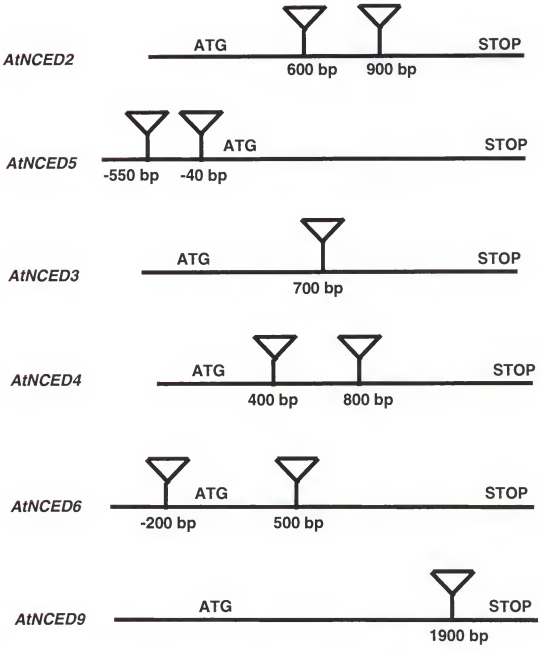


Fig. 4-5. Schematic diagram showing the positions of T-DNA insertions within *AtNCED2, 3, 4, 5, 6* and *Ds* insertion within *AtNCED9*.

down to individual plants. Positive candidates have been identified for all the other *AtNCEDs* in the first two rounds of screening (Fig. 4-5), and the third round of screening is in progress.

***AtNCED2* knockout mutants**

The AtNCED2 has 90% amino acid similarity to VP14. Southern analysis revealed 10 positive PCR products which were amplified from 3'*AtNCED2* primer and T-DNA left border primer JL202 in the first round of PCR screening that performed on 30 super pools of 2025 T-DNA lines (Fig 4-6, A). The sizes of two PCR positives are larger than 3 kb (AtNCED2 gene is 1.8 kb) and the T-DNA may be inserted into the gene upstream of *AtNCED2*. The sizes of 6 positive PCR products are 1.6 kb, cloning and sequencing four of them revealed that they were PCR artifacts. The remaining two PCR products, 1.2 kb and 0.9 kb, were identified in pool X9 and pool X24. Cloning and sequencing of these two products showed that one has a T-DNA insertion 600 bp downstream of ATG and the other one has a T-DNA insertion 900 bp downstream of ATG. No positives were identified on PCR products amplified with 5'*AtNCED2* and JL202 primers. The second round of PCR screening was performed on 18 sub pools each containing 225 T-DNA lines derived from pools of X9 and X24. The two positives were narrowed down to pool H74 and pool H81, respectively. The third round of screening was carried out on 50 pools each containing nine T-DNA lines, and the positives were narrowed down to pools CSH 1834 and CSH 5181, each containing nine T-DNA lines. Seed pools of CSH 1834 and CSH 5181 were germinated and plants were grown up. PCR reactions were performed on DNA extracted from 60 randomly selected individual plants

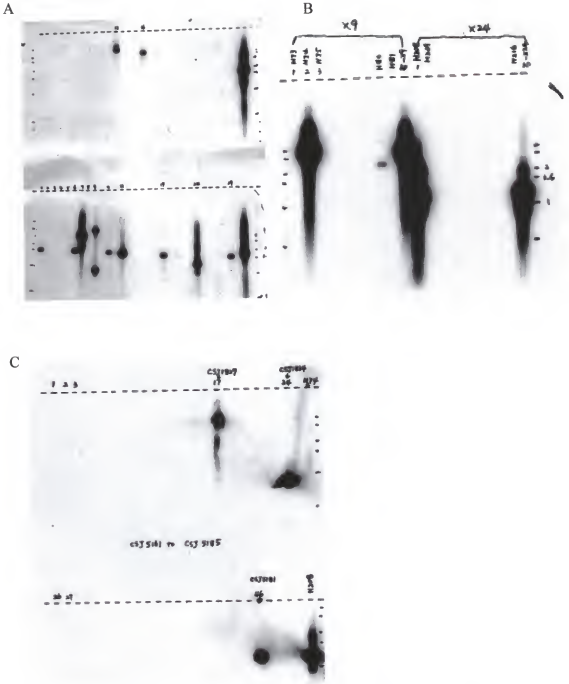


Fig. 4-6. Southern blots of PCR products amplified from T-DNA pools with AtNCED2 5' or 3' primers combined with JL202 primer. **A.** Southern blot of 30 pools of 2025. **B.** Southern blot of 18 pools of 225. C. Southern blot of 50 pools of 9.

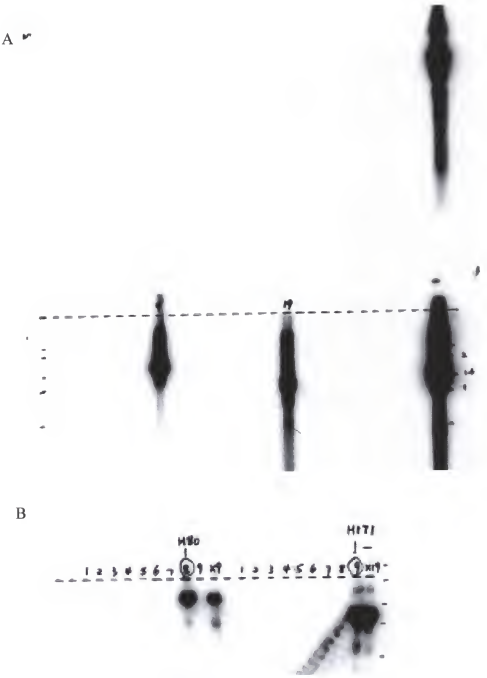


Fig. 4-7. Southern blots of PCR products amplified from T-DNA pools with AtNCED5 5' or 3' primers combined with and JL202 primer. A. Southern blot of 30 pools of 2025. B. Southern blot of 18 pools of 225.

from CSH 1834 and CSH 5181. One positive plant was identified from CSH 1834 and five positive plants were identified from CSH 5181.

***AtNCED5* knockout mutants**

Two positive PCR products were identified in super pool X9 and pool X19 with 3' AtNCED5 and JL202 primers (Fig. 4-7). Cloning and sequencing of the PCR products showed that one has a T-DNA insertion 40 bp upstream of ATG and the other one has a T-DNA insertion 550 bp upstream of ATG. These two positives were narrowed down to pool H80 and H171 in the second round of screening, and they were narrowed down to CSH 4250 and CSH 1981 in the third round of screening. One positive individual plant was identified from 30 randomly selected plants from CSH 1981 and five positive individual plants were identified from 30 randomly selected plants from CSH 4250.

Discussion

Does ABA Play a Role in Lateral Root Initiation?

AtNCED2-GUS transgenic plants showed GUS staining in the pericycle cells that surround lateral roots initials, and the staining can be induced by IAA. This pattern of expression is suggestive of a ABA function in lateral root initiation. Although there has not been any documented evidence showing that ABA is required for lateral root initiation, it was observed that the *aba3* mutant of Arabidopsis failed to induce lateral roots (Dr. Jocelyn Malamyl, per comm). In addition, 35S-AtNCED2 transgenic plants showed inhibited lateral root growth, although it is unknown whether the inhibition is due to ABA overexpression or cosuppression of the endogenous *AtNCED2* gene in the roots of transgenic plants. No obvious lateral root phenotype was observed in *AtNCED2*

knockout mutants. One possibility is that AtNCED2 only accounts for a subset of root source ABA. Another possibility is that more sensitive tests are needed to compare the frequency and growth rate of lateral root initials and the root response to different concentrations of IAA in the wildtype and the mutant plants.

The induction of AtNCED2-GUS staining by IAA in lateral root initials suggests that *AtNCED2* promoter may be regulated by auxin. Inspection of the *AtNCED2* promoter did not reveal a typical AuxRE (TGTCTC) recognized by ARF transcription factors (Ulmasov et al. 1997). A study of ectopically expressed VP1 in Arabidopsis revealed that VP1 is also conditioned by auxin signaling in roots (Suzuki, et al. unpublished data), thus raises a possibility that *AtNCED2* promoter may be regulated by VP1-like proteins. It is also possible that auxin indirectly regulates *AtNCED2*: the *AtNCED2* induction by auxin is only an indirect effect of auxin induction of lateral root initiation. However, the GUS staining in the pericycle cells distal to the elongation zone appears to predict the positions of new lateral root initials, and this AtNCED2 induction appears to be an early event in the lateral root initiation.

ABA Function in Vegetative Growth and Flower Development

The role of ABA in vegetative growth and flower formation is poorly understood. The AtNCED2 overexpression transgenic plants showed delayed vegetative development and male sterility. The delayed vegetative growth includes cotyledon emergence, rosette leave growth, and flowering time. The severity of the phenotype is correlated well with transcriptional levels of the transgene. The phenotype suggests that overproduction of ABA inhibits vegetative growth and development. Although there is not any large increase in the bulk ABA content in transgenic lines, it is possible that the localized

cellular ABA levels are much higher in tissues responsible for the transgenic plant phenotype. Another possibility is that the key regulatory enzyme in the ABA degradation pathway, 8'-hydroxylase, is upregulated by ABA (Cutler et al. 1997), therefore, the overproduced ABA in transgenic plants may be rapidly degraded by constitutive induction of 8'-hydroxylase.

The levels of ABA increase when plants suffer stresses. The pigmentation accumulation phenotype is intriguing since it mimics the plant phenotype grown in the stressed condition, such as strong light or drought. In addition, a good correlation was observed between pigmentation accumulation and sterility. The more heavily pigmented plants showed more severe sterility, as well as the transgene expression levels.

In summary, The AtNCED2 promoter-GUS staining pattern and the AtNCED2 overexpression transgenic plants phenotype suggest that ABA may play roles in lateral root initiation, in shoot and flower development. Characterization of the knockout mutants of gene family members will help in determining the physiological and biochemical functions and each family members and the ABA functions. Due to substantial genetic redundancy, double or triple mutants may need to be generated to see a discernible phenotype. Once such mutant is obtained, it can be served as a transformation host to test which member of the AtNCEDs can complement the phenotype, and thus actually functions in the ABA biosynthesis.

SUMMARY AND CONCLUSIONS

The key regulatory enzyme in the abscisic acid (ABA) biosynthetic pathway, *9-cis* epoxycarotenoid dioxygenase (NCED), is encoded by a complex gene family. In this work, it is shown that there are nine putative NCED-like genes in Arabidopsis (AtNCED). *AtNCED2, 3, 5, 9* show high sequence homology to *NCEDs* from other species, thus are very likely to function as NCED in Arabidopsis. The other AtNCEDs may catalyze other carotenoid cleavage reactions. Northern analysis revealed that these genes are differentially expressed in leaves and roots, and induced by water stress. Chloroplast import assays showed that AtNCED1, 2, 4 and 5 proteins are differentially localized suggesting that accessing substrates, therefore enzyme activity, may be regulated at the post-translational level. The AtNCED2 promoter-GUS staining pattern and the AtNCED2 overexpression plant phenotype suggest that AtNCED2 may have a function in lateral root initiation and flower development.

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BIOGRAPHICAL SKETCH

Wen-Tao Deng was born on September 14 1969 in Gansu Province, China. She completed high school in 1987. She enrolled in Lanzhou University and received her B. S. in Plant Physiology in 1991. In the following two years, she worked at the Beijing Institute of Botany, Academia Sinica as a graduate student. She moved to Gainesville, Florida in 1993 to join her husband who was a graduate student in the Plant Molecular and Cellular Biology program. She started her Master studies in the Plant Molecular and Cellular Biology program at the University of Florida in January 1995 and began her doctoral studies in 1996.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Donald R. McCarty, Chair
Professor of Horticultural Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Karen E. Koch
Professor of Horticultural Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

William B. Gurley
Professor of Microbiology and
Cell Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

John M. Davis
Associate Professor of Forest Resources and
Conservation

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Associate Professor of Chemistry

This dissertation was submitted to the Graduate Faculty of the interdisciplinary program in Plant Molecular and Cellular Biology in the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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